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(54) Title: HAMMERHEAD RIBOZYMES WITH EXTENDED CLEAVAGE RULE			
(57) Abstract <p>Disclosed are compositions having an RNA-cleavage activity, as well as their use for cleaving RNA-substrates <i>in vitro</i> and <i>in vivo</i>. The compositions contain an active center, the subunits of which are selected from nucleotides and/or nucleotide analogues, as well as flanking regions contributing to the formation of a specific hybridization with an RNA substrate. Preferred compositions form, in combination with an RNA substrate, a structure resembling a hammerhead structure. The active center of the disclosed compositions is characterized by the presence of I^{15.1} which allows cleavage of RNA substrates having C^{16.1}.</p>			

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HAMMERHEAD RIBOZYMES WITH EXTENDED CLEAVAGE RULE

Background of the Invention

The present invention is in the field of compositions having RNA-cleavage activity.

5 Hammerhead ribozymes are an example of catalytic RNA molecules which are able to recognize and cleave a given specific RNA substrate (Hotchins *et al.*, *Nucleic Acids Res.* 14:3627 (1986); Keese and Symons, in *Viroids and viroid - like pathogens* (J.J. Semanchik, publ., CRC-Press, Boca Raton, Florida, 1987), pages 1-47). The catalytic center of hammerhead
10 ribozymes is flanked by three stems and can be formed by adjacent sequence regions of the RNA or also by regions which are separated from one another by many nucleotides. Figure 1 shows a diagram of such a catalytically active hammerhead structure. The stems have been denoted I, II and III. The nucleotides are numbered according to the standard nomenclature for
15 hammerhead ribozymes (Hertel *et al.*, *Nucleic Acids Res.* 20:3252 (1992)). In this nomenclature, bases are denoted by a number which relates their position relative to the 5' side of the cleavage site. Furthermore, each base that is involved in a stem or loop region has an additional designation (which is denoted by a decimal point and then another number) that defines the
20 position of that base within the stem or loop. A designation of N^{11.3} would indicate that this base is involved in a paired region and that it is the third base in that stem going away from the core region. This accepted convention for describing hammerhead derived ribozymes allows for the nucleotides involved in the core of the enzyme to always have the same
25 number relative to all of the other nucleotides. The size of the stems involved in substrate binding or core formation can be any size and of any sequence, and the position of A⁹, for example, will remain the same relative to all of the other core nucleotides. Nucleotides designated, for example, N^{^12} or N^{9^} represent an inserted nucleotide where the position of the caret
30 (^) relative to the number denotes whether the insertion is before or after the indicated nucleotide. Thus, N^{^12} represents a nucleotide inserted before

nucleotide position 12, and N^{9^*} represents a nucleotide inserted after nucleotide position 9.

The consensus sequence of the catalytic core structure is described by Ruffner and Uhlenbeck (*Nucleic Acids Res.* 18:6025-6029 (1990)). Perriman
5 *et al.* (*Gene* 113:157-163 (1992)) have meanwhile shown that this structure can also contain variations, for example, naturally occurring nucleotide insertions such as N^{9^*} and N^{12} . Thus, the positive strand of the satellite RNA of the tobacco ring-spot virus does not contain any of the two nucleotide insertions while the +RNA strand of the virusoid of the lucerne
10 transient streak virus (vLTSV) contains a $N^{9^*} = U$ insertion which can be mutated to C or G without loss of activity (Sheldon and Symons, *Nucleic Acids Res.* 17:5679-5685 (1989)). Furthermore, in this special case, $N^7 = A$ and $R^{15.1} = A$. On the other hand, the minus strand of the carnation stunt associated viroid (- CarSV) is quite unusual since it contains both nucleotide
15 insertions, that is $N^{12} = A$ and $N^{9^*} = C$ (Hernandez *et al.*, *Nucleic Acids Res.* 20:6323-6329 (1992)). In this viroid $N^7 = A$ and $R^{15.1} = A$. In addition, this special hammerhead structure exhibits a very effective self-catalytic cleavage despite the more open central stem.

Possible uses of hammerhead ribozymes include, for example,
20 generation of RNA restriction enzymes and the specific inactivation of the expression of genes in, for example, animal, human or plant cells and prokaryotes, yeasts and plasmodia. A particular biomedical interest is based on the fact that many diseases, including many forms of tumors, are related to the overexpression of specific genes. Inactivating such genes by cleaving
25 the associated mRNA represents a possible way to control and eventually treat such diseases. Moreover there is a great need to develop antiviral, antibacterial, and antifungal pharmaceutical agents. Ribozymes have potential as such anti-infective agents since RNA molecules vital to the survival of the organism can be selectively destroyed.

30 One of the greatest impediments to using hammerhead based ribozymes for pharmaceutical agents is the limited availability of acceptable targets in normal Gerlach designs and when targeting pre-formed half

hammerhead structures (see WO 97/18312). In addition to needing the correct hybridizing sequences for substrate binding, substrates for hammerhead ribozymes have been shown to strongly prefer the triplet $N^{16.2}U^{16.1}H^{17}$ where N can be any nucleotide, U is uridine, and H is either adenosine, cytidine, or uridine (Koizumi *et al.*, *FEBS Lett.* 228, 228-230 (1988); Ruffner *et al.*, *Biochemistry* 29, 10695-10702 (1990); Perriman *et al.*, *Gene* 113, 157-163 (1992)). The fact that changes to this general rule for substrate specificity result in non-functional substrates implies that there are "non core compatible" structures which are formed when substrates are provided which deviate from the stated requirements. Evidence along these lines was recently reported by Uhlenbeck and co-workers (*Biochemistry* 36:1108-1114 (1997)) when they demonstrated that the substitution of a G at position 17 caused a functionally catastrophic base pair between G¹⁷ and C³ to form, both preventing the correct orientation of the scissile bond for cleavage and the needed tertiary interactions of C³ (Murray *et al.*, *Biochem. J.* 311:487-494 (1995)). The strong preference for a U at position 16.1 may exist for similar reasons. Many experiments have been done in an attempt to isolate ribozymes which are able to efficiently relieve the requirement of a U at position 16.1, however, attempts to find hammerhead type ribozymes which can cleave substrates having a base other than a U at position 16.1 have proven impossible (Perriman *et al.*, *Gene* 113, 157-163 (1992)).

Efficient catalytic molecules with reduced or altered requirements in the cleavage region are highly desirable because their isolation would greatly increase the number of available target sequences that molecules of this type could cleave. For example, it would be desirable to have a ribozyme variant that could efficiently cleave substrates containing triplets other than $N^{16.2}U^{16.1}H^{17}$ since this would increase the number of potential target cleavage sites.

Chemically modified oligonucleotides which contain a block of deoxyribonucleotides in the middle region of the molecule have potential as pharmaceutical agents for the specific inactivation of the expression of genes (Giles *et al.*, *Nucleic Acids Res.* 20:763-770 (1992)). These oligonucleotides

can form a hybrid DNA-RNA duplex in which the DNA bound RNA strand is degraded by RNase H. Such oligonucleotides are considered to promote cleavage of the RNA and so cannot be characterized as having an RNA-cleaving activity nor as cleaving an RNA molecule (the RNase H is
5 cleaving). A significant disadvantage of these oligonucleotides for *in vivo* applications is their low specificity, since hybrid formation, and thus cleavage, can also take place at undesired positions on the RNA molecules.

Previous attempts to recombinantly express catalytically active RNA molecules in the cell by transfecting the cell with an appropriate gene have
10 not proven to be very effective since a very high expression was necessary to inactivate specific RNA substrates. In addition the vector systems which are available now cannot generally be applied. Furthermore, unmodified ribozymes cannot be administered directly due to the sensitivity of RNA to degradation by RNases and their interactions with proteins. Thus, chemically
15 modified active substances have to be used in order to administer hammerhead ribozymes exogenously (discussed, for example, by Heidenreich *et al.*, *J. Biol. Chem.* 269:2131-2138 (1994); Kiehnopf *et al.*, *EMBO J.* 13:4645-4652 (1994); Paoletta *et al.*, *EMBO J.* 11:1913-1919 (1992); and Usman *et al.*, *Nucleic Acids Symp. Ser.* 31:163-164 (1994)).

20 U.S. Pat. No. 5,334,711 describes such chemically modified active substances based on synthetic catalytic oligonucleotide structures with a length of 35 to 40 nucleotides which are suitable for cleaving a nucleic acid target sequence and contain modified nucleotides that contain an optionally substituted alkyl, alkenyl or alkynyl group with 1 - 10 carbon atoms at the
25 2'-O atom of the ribose. These oligonucleotides contain modified nucleotide building blocks and form a structure resembling a hammerhead structure. These oligonucleotides are able to cleave specific RNA substrates.

The use of a large number of deoxyribonucleotides in the hybridization arms or in the active center can lead to a loss of specificity due
30 to an activation of RNase H since sequences which are related to the desired target sequence can also be cleaved. Moreover, catalytic DNA oligomers are

not particularly well suited for *in vivo* applications due to interactions with proteins, and lack of resistance to degradation by nucleases.

It is therefore an object of the present invention to provide compositions that cleave RNA, and in particular to provide RNA-cleaving oligomers which at the same time have a high stability, activity, and specificity.

It is another object of the present invention to provide compositions that cleave RNA substrates having a cleavage site triplet other than $N^{16.2}U^{16.1}H^{17}$.

10 Summary of the Invention

Disclosed are compositions having an RNA-cleavage activity, as well as their use for cleaving RNA substrates *in vitro* and *in vivo*. The compositions contain an active center, the subunits of which are selected from nucleotides and/or nucleotide analogues, as well as flanking regions contributing to the formation of a specific hybridization with an RNA substrate. Preferred compositions form, in combination with an RNA substrate, a structure resembling a hammerhead structure. The active center of the disclosed compositions is characterized by the presence of $I^{15.1}$ which allows cleavage of RNA substrates having $C^{16.1}$.

20 Brief Description of the Drawings

Figure 1 is a diagram of a hammerhead structure and the corresponding nomenclature (SEQ ID NO:1). Cleavage occurs between H^{17} and $N^{1.1}$ to generate the 2',3'-cyclic phosphate at H^{17} .

Figure 2 is a diagram of an RNA substrate (SEQ ID NO:3) in association with an example of an oligomer (SEQ ID NO:2) that cleaves the RNA substrate. The structure formed by the oligomer and the substrate resembles the structure of a hammerhead ribozyme. In this case, the substrate makes up half of stems I and III, and loops I and III are not present. Cleavage occurs 3' of H^{17} .

Figure 3 is a diagram showing the interaction of the $A^{15.1}-U^{16.1}$ base pair in hammerhead ribozymes (top), and the predicted isostructural

interaction of a $I^{15.1}$ - $C^{16.1}$ base pair (bottom) that replaces the $A^{15.1}$ - $U^{16.1}$ base pair.

Figure 4A is a graph of fraction of cleavage product versus time (in minutes) for the cleavage of a short 5'-fluorescein labelled oligoribonucleotide substrate (SEQ ID NO:8) containing a GCA site by four variants of 2'-O-allylated 5-ribo catalytic oligomers each containing a different nucleobase at position N^7 (U, C, A and G; SEQ ID NOS:18, 22, 23, and 24, respectively).

Figure 4B is a graph of fraction of cleavage product versus time (in minutes) for the cleavage of a short 5'-fluorescein labelled oligoribonucleotide substrate of SEQ ID NO:8 containing a GCA site by four variants of 2'-O-allylated 5-ribo catalytic oligomers each containing a different nucleobase or base analogue at position N^7 (U, 5-nitroindole, I, and quinazoline-2, 4-dione; SEQ ID NOS:18, 27, 25, and 26, respectively).

Detailed Description of the Invention

Disclosed are compositions having an RNA-cleavage activity, as well as their use for cleaving RNA-substrates *in vitro* and *in vivo*. The compositions contain an active center, the subunits of which are selected from nucleotides and/or nucleotide analogues, as well as flanking regions contributing to the formation of a specific hybridization with an RNA substrate. Preferred compositions form, in combination with an RNA substrate, a structure resembling a hammerhead structure. The active center of the disclosed compositions is characterized by the presence of $I^{15.1}$ which allows cleavage of RNA substrates having $C^{16.1}$.

All naturally occurring hammerhead ribozymes have an $A^{15.1}$ - $U^{16.1}$ base pair. In addition, it is known that substrates for ribozymes based on the consensus hammerhead sequence strongly prefer a substrate that contains an $N^{16.2}U^{16.1}H^{17}$ triplet in which H^{17} is not a guanosine (Koizumi *et al.*, *FEBS Lett.* 228, 228-230 (1988); Ruffner *et al.*, *Biochemistry* 29, 10695-10702 (1990); Perriman *et al.*, *Gene* 113, 157-163 (1992)). Many experiments have been done in an attempt to isolate ribozymes which are able to efficiently relieve the requirement of a U at position 16.1, however, attempts to find

ribozymes which can cleave substrates having a base other than a U at position 16.1 have proven impossible (Perriman *et al.*, *Gene* 113, 157-163 1992, Singh *et al.*, *Antisense and Nucleic Acid Drug Development* 6:165-168 (1996)).

5 However, examination of the recently published X-ray crystal structures (Pley *et al.*, *Nature* 372:68-74 (1994), Scott *et al.*, *Cell* 81:991-1002 (1995), and Scott *et al.*, *Science* 274:2065-2069 (1996)) led to the realization that the A^{15.1}-U^{16.1} interaction is a non-standard base pair with a single hydrogen bond between the exocyclic amine (N6) of the adenosine and
10 the 4-oxo group of the uridine. Modeling studies (based on the crystal structure) then led to the discovery that the interaction of the wild-type A^{15.1}-U^{16.1} base pair can be spatially mimicked by replacement with an I^{15.1}-C^{16.1} base pair that adopts an isostructural orientation and which preserves the required contact of the 2-keto group of C^{16.1} with A⁶ of the uridine turn. In
15 the model, the polarity of the stabilizing hydrogen bond between positions 15.1 and 16.1 is reversed in the I^{15.1}-C^{16.1} interaction, but the correct orientation of the bases around this bond is maintained.

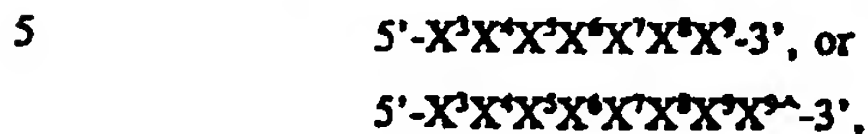
 It has been discovered that Gerlach type ribozyme analogues containing an inosine at position 15.1 readily cleave RNA substrates
20 containing an N^{16.2}C^{16.1}H¹⁷ triplet. Based on this, disclosed are compositions, preferably synthetic oligomers, which cleave a nucleic acid target sequence containing the triplet N^{16.2}C^{16.1}H¹⁷. It is preferred that H¹⁷ is not guanosine. The ability to cleave substrates having N^{16.2}C^{16.1}X¹⁷ triplets effectively doubles the number of targets available for cleavage by compositions of the type
25 disclosed.

Compositions Having an RNA-cleavage Activity

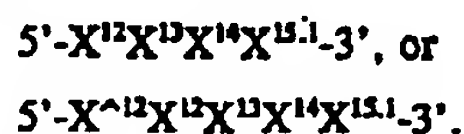
 Specifically disclosed is a composition that cleaves an RNA substrate, where the composition includes components (a) and (b), where component (a) includes a structure 5'-Z₁-Z₂-3' and component (b) includes a structure
30 5'-Z₃-Z₄-3'. Components (a) and (b) can either be separate molecules or can be covalently coupled. Elements Z₁ and Z₄ in components (a) and (b) are each oligomeric sequences which are made up of nucleotides, nucleotide

analogues, or a combination of both, or are oligonucleotide analogues. The oligomeric sequences of elements Z_1 and Z_4 specifically interact with the RNA substrate, preferably by hybridization.

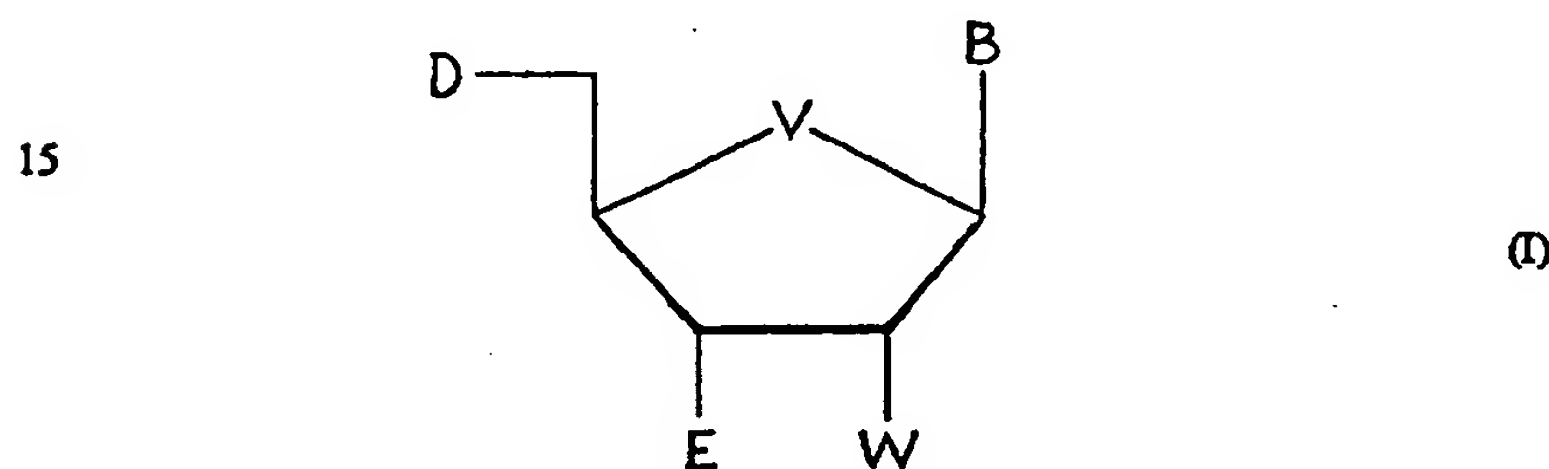
In these preferred compositions, element Z_2 has a structure of



and element Z_3 has a structure of



10 Elements Z_1 and Z_3 in these preferred compositions are made up of nucleotides, nucleotide analogues, or a combination of both. The nucleotides and nucleotide analogues in elements Z_1 and Z_3 each have the structure



20 In structure (I) each B can be adenin-9-yl, cytosin-1-yl, guanin-9-yl, uracil-1-yl, uracil-5-yl, hypoxanthin-9-yl, thymine-1-yl, 5-methylcytosin-1-yl, 2,6-diaminopurin-9-yl, purin-9-yl, 7-deazaadenin-9-yl, 7-deazaguanin-9-yl, 5-propynylcytosin-1-yl, 5-propynyluracil-1-yl, isoguanin-9-yl, 2-aminopurin-9-yl, 6-methyluracil-1-yl, 4-thiouracil-1-yl, 2-pyrimidone-1-yl, quinazoline-2,4-dione-1-yl, xanthin-9-yl, N²-dimethylguanin-9-yl or a functional equivalent thereof;

Each V can be an O, S, NH, or CH₂ group.

Each W can be -H, -OH, -COOH, -CONH₂, -CONHR¹, -CONR¹R², -NH₂, -NHR¹, -NR¹R², -NHCOR¹, -SH, SR¹, -F, -ONH₂, -ONHR¹, -ONR¹R², -NHOH, -NHOR¹, -NR²OH, -NR²OR¹, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkyl, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyl, substituted or unsubstituted C₃-C₁₀ straight

chain or branched alkynyl, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkoxy, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyloxy, and substituted or unsubstituted C₂-C₁₀ straight chain or branched alkynyloxy. The substituents for W groups are independently
 5 halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto. R¹ and R² can be substituted or unsubstituted alkyl, alkenyl, or alkynyl groups, where the substituents are independently halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto.

D and E are residues which together form a phosphodiester or
 10 phosphorothioate diester bond between adjacent nucleosides or nucleoside analogues or together form an analogue of an internucleosidic bond.

B is hypoxanthin-9-yl, or a functional equivalent thereof, in X^{12.1}; B can be guanine-9-yl, hypoxanthin-9-yl or 7-deazaguanine-9-yl in X³, X⁴, and X¹²; B can be adenine-9-yl, 2,6-diaminopurine-9-yl, purine-9-yl or 7-deazaadenine-9-yl in X⁶, X⁹, X¹³, and X¹⁴; B can be uracil-1-yl, uracil-5-yl, thymine-1-yl or 5-propynyluracil-1-yl in X⁴; B can be cytosine-1-yl, 5-methylcytosine-1-yl or 5-propynylcytosine-1-yl in X³; and B can be adenine-9-yl, cytosine-1-yl, guanine-9-yl, uracil-1-yl, uracil-5-yl, hypoxanthin-9-yl, thymine-1-yl, 5-methylcytosine-1-yl, 2,6-diaminopurine-9-yl, purine-9-yl, 7-deazaadenine-9-yl, 7-deazaguanine-9-yl, 5-propynylcytosine-1-yl, 5-propynyluracil-1-yl, isoguanine-9-yl, 2-aminopurine-9-yl, 6-methyluracil-1-yl, 4-thiouracil-1-yl, 2-pyrimidone-1-yl, quinazoline-2,4-dione-1-yl, xanthine-9-yl, N²-dimethylguanine-9-yl, or a functional equivalent thereof in X⁷, X⁸, and X¹¹. B of X^{15.1} is preferably hypoxanthin-9-yl or an analog where no hydrogen bond can form between
 20 any group at the 2 position of the base and the 2-oxo group of C^{16.1}.
 25 Preferably, B is not guanine-9-yl in X^{15.1}.

B in X³, X⁴, X⁵, X⁶, X⁸, X⁹, X¹², X¹³, and X¹⁴ can also be a functionally equivalent nucleobase within the context of the catalytic core of a hammerhead ribozyme. For example, C³, U⁴, G⁵, and A⁶ in hammerhead
 30 ribozymes form a structure closely resembling a uridine turn in a tRNA (Pley *et al.*, *Nature* 372:68-74 1994). Other groups of nucleotides can also form uridine turns and so nucleotides X³, X⁴, X⁵, X⁶ may be replaced as a group

with nucleotides or nucleotide analogues that have the potential to form a structure resembling a uridine turn. Similarly, the sheared base pairs in the catalytic core of hammerhead ribozymes have interactions that may be similar to interactions of other non-canonical base pairs. Knowledge of the crystal
5 structure of the catalytic core of hammerhead ribozymes, combined with the discovery that a Gerlach type hammerhead ribozyme in which a non-canonical base pair has been replaced with an isostructural non-canonical base pair is active, indicates that analogous isostructural base pair replacements should be possible elsewhere in the catalytic core.

10 **Definitions**

As used herein, oligomer refers to oligomeric molecules composed of subunits where the subunits can be of the same class (such as nucleotides) or a mixture of classes (such as nucleotides and ethylene glycol). It is preferred that the disclosed oligomers be oligomeric sequences, non-nucleotide linkers,
15 or a combination of oligomeric sequences and non-nucleotide linkers. It is more preferred that the disclosed oligomers be oligomeric sequences. Oligomeric sequences are oligomeric molecules where each of the subunits includes a nucleobase (that is, the base portion of a nucleotide or nucleotide analogue) which can interact with other oligomeric sequences in a base-
20 specific manner. The hybridization of nucleic acid strands is a preferred example of such base-specific interactions. Oligomeric sequences preferably are comprised of nucleotides, nucleotide analogues, or both, or are oligonucleotide analogues.

Non-nucleotide linkers can be any molecule, which is not an
25 oligomeric sequence, that can be covalently coupled to an oligomeric sequence. Preferred non-nucleotide linkers are oligomeric molecules formed of non-nucleotide subunits. Examples of such non-nucleotide linkers are described by Letsinger and Wu, (*J. Am. Chem. Soc.* 117:7323-7328 (1995)), Benseler *et al.*, (*J. Am. Chem. Soc.* 115:8483-8484 (1993)) and Fu *et al.*,
30 (*J. Am. Chem. Soc.* 116:4591-4598 (1994)). Preferred non-nucleotide linkers, or subunits for non-nucleotide linkers, include substituted or unsubstituted C₁-C₁₀ straight chain or branched alkyl, substituted or

unsubstituted C₂-C₁₀ straight chain or branched alkenyl, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkynyl, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkoxy, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyloxy, and substituted or
5 unsubstituted C₂-C₁₀ straight chain or branched alkynyloxy. The substituents for these preferred non-nucleotide linkers (or subunits) can be halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto.

As used herein, nucleoside refers to adenosine, guanosine, cytidine, uridine, 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-
10 deoxycytidine, or thymidine. A nucleoside analogue is a chemically modified form of nucleoside containing a chemical modification at any position on the base or sugar portion of the nucleoside. As used herein, the term nucleoside analogue encompasses, for example, both nucleoside analogues based on naturally occurring modified nucleosides, such as inosine and pseudouridine,
15 and nucleoside analogues having other modifications, such as modifications to the 2' position of the sugar. As used herein, nucleotide refers to a phosphate derivative of nucleosides as described above, and a nucleotide analogue is a phosphate derivative of nucleoside analogues as described above. The subunits of oligonucleotide analogues, such as peptide nucleic acids, are also
20 considered to be nucleotide analogues.

As used herein, a ribonucleotide is a nucleotide having a 2' hydroxyl function. Analogously, a 2'-deoxyribonucleotide is a nucleotide having only 2' hydrogens. Thus, ribonucleotides and deoxyribonucleotides as used herein refer to naturally occurring nucleotides having nucleoside components
25 adenosine, guanosine, cytidine, and uridine, or 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine, and thymidine, respectively, without any chemical modification. Ribonucleosides, deoxyribonucleosides, ribonucleoside analogues and deoxyribonucleoside analogues are similarly defined except that they lack the phosphate group, or an analogue of the
30 phosphate group, found in nucleotides and nucleotide analogues.

As used herein, oligonucleotide analogues are polymers of nucleic acid-like material with nucleic acid-like properties, such as sequence

dependent hybridization, that contain at one or more positions, a modification away from a standard RNA or DNA nucleotide. A preferred example of an oligonucleotide analogue is peptide nucleic acid.

As used herein, base pair refers to a pair of nucleotides or nucleotide analogues which interact through one or more hydrogen bonds. The term
5 base pair is not limited to interactions generally characterized as Watson-Crick base pairs, but includes non-canonical or sheared base pair interactions (Topal and Fresco, *Nature* 263:285 (1976); Lomant and Fresco, *Prog. Nucl. Acid Res. Mol. Biol.* 15:185 (1975)). Thus, nucleotides A^{13.1} and U^{16.1} form
10 a base pair in hammerhead ribozymes (see Figure 1) but the base pair is non-canonical (see Figure 3).

The internucleosidic linkage between two nucleosides can be achieved by phosphodiester bonds or by modified phospho bonds such as by phosphorothioate groups or other bonds such as, for example, those described
15 in U.S. Pat. No. 5,334,711.

Flanking Elements Z₁ and Z₄

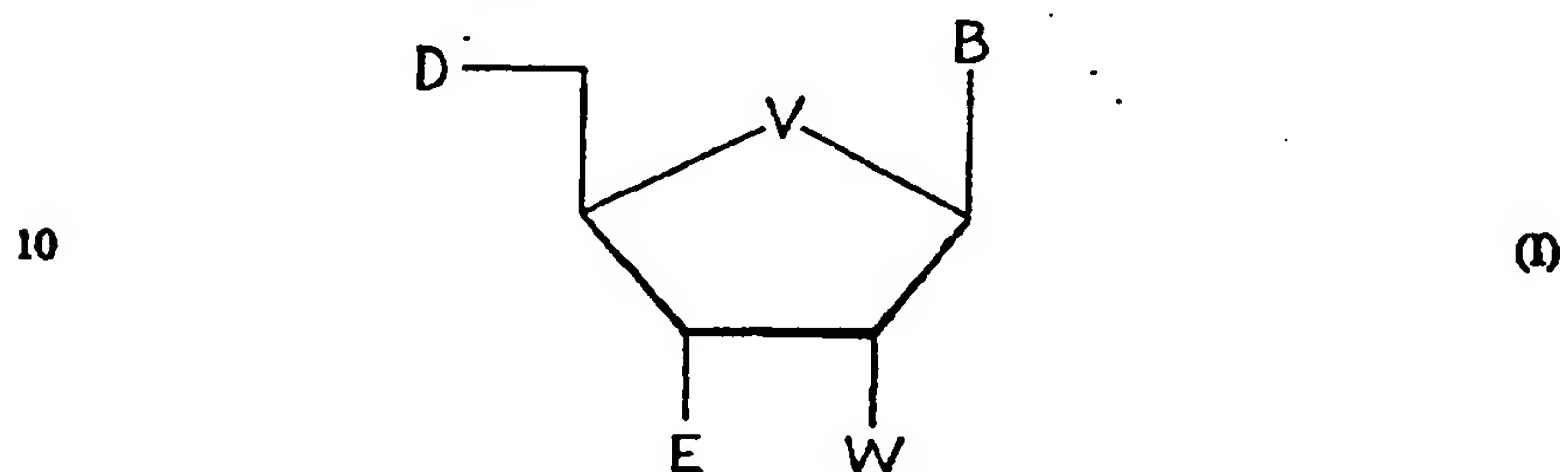
The monomeric subunits of elements Z₁ and Z₄ which flank the active center (formed by elements Z₂ and Z₃) are preferably nucleotides and/or nucleotide analogues. Elements Z₁ and Z₄ are designed so that they
20 specifically interact, preferably by hybridization, with a given RNA substrate and, together with the active center Z₂ and Z₃, form a structure (preferably a structure resembling that of a hammerhead ribozyme) which specifically cleaves the RNA substrate.

The subunits of elements Z₁ and Z₄ can, on the one hand, be
25 ribonucleotides. However, it is preferred that the number of ribonucleotides be as small as possible since the presence of ribonucleotides reduces the *in vivo* stability of the oligomers. Elements Z₁ and Z₄ (and also the active center Z₂ and Z₃) preferably do not contain any ribonucleotides at the positions containing pyrimidine nucleobases. Such positions preferably
30 contain nucleotide analogues.

The use of a large number of deoxyribonucleotides in elements Z₁ and Z₄ is also less preferred since undesired interactions with proteins can occur

or an unintended RNase H-sensitive DNA-RNA hybrid could form. Thus, elements Z_1 and Z_4 each preferably contain (1) no ribonucleotides, and (2) no sequences of more than 3 consecutive deoxyribonucleotides.

The subunits of elements Z_1 and Z_4 are preferably nucleotides, nucleotide analogues, or a combination. Preferably, the nucleotides and nucleotide analogues in elements Z_1 and Z_4 each have the structure



In structure (I) each B can be adenin-9-yl, cytosin-1-yl, guanin-9-yl, uracil-1-yl, uracil-5-yl, hypoxanthin-9-yl, thymine-1-yl, 5-methylcytosin-1-yl, 2,6-diaminopurin-9-yl, purin-9-yl, 7-deazaadenin-9-yl, 7-deazaguanin-9-yl, 5-propynylcytosin-1-yl, 5-propynyluracil-1-yl, isoguanin-9-yl, 2-aminopurin-9-yl, 6-methyluracil-1-yl, 4-thiouracil-1-yl, 2-pyrimidone-1-yl, quinazoline-2,4-dione-1-yl, xanthin-9-yl, N^2 -dimethylguanin-9-yl or a functional equivalent thereof;

Each V can be an O, S, NH, or CH_2 group.

Each W can be -H, -OH, -COOH, -CONH₂, -CONHR¹, -CONR¹R², -NH₂, -NHR¹, -NR¹R², -NHCOR¹, -SH, SR¹, -F, -ONH₂, -ONHR¹, -ONR¹R², -NHOH, -NHOR¹, -NR²OH, -NR²OR¹, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkyl, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyl, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkynyl, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkoxy, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyloxy, and substituted or unsubstituted C₂-C₁₀ straight chain or branched alkynyloxy. The substituents for W groups are independently halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto. R¹ and R² can be substituted or unsubstituted alkyl, alkenyl, or

alkynyl groups, where the substituents are independently halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto.

D and E are residues which together form a phosphodiester or phosphorothioate diester bond between adjacent nucleosides or nucleoside analogues or together form an analogue of an internucleosidic bond.

For elements Z_1 and Z_4 having nucleotide and/or nucleotide analogues of structure (I), it is preferred that each W is substituted or unsubstituted C_1 - C_{10} straight chain or branched alkoxy, C_1 - C_{10} straight chain or branched alkenyloxy, or C_1 - C_{10} straight chain or branched alkynyloxy.

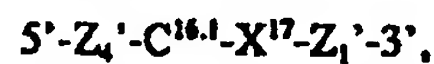
In addition, the flanking elements Z_1 and Z_4 can also contain nucleotide analogues such as peptide nucleic acids (also referred to as peptidic nucleic acids; see for example Nielsen *et al.*, *Science* 254:1497-1500 (1991), and Ducholm *et al.*, *J. Org. Chem.* 59:5767-5773 (1994)). In this case the coupling of individual subunits can, for example, be achieved by acid amide bonds. Elements Z_1 and Z_4 , when based on peptide nucleic acids, can be coupled to elements Z_2 and Z_3 , based on nucleotides or nucleotide analogues, using either suitable linkers (see, for example, Petersen *et al.*, *BioMed. Chem. Lett.* 5:1119-1121 (1995)) or direct coupling (Bergmann *et al.*, *Tetrahedron Lett.* 36:6823-6826 (1995)). Where elements Z_1 and Z_4 contain a combination of nucleotides (and/or nucleotide analogues) and peptide nucleic acid, similar linkages can be used to couple the different parts.

The subunits of the flanking elements Z_1 and Z_4 contain nucleobases or nucleobase analogues which can hybridize or interact with nucleobases that occur naturally in RNA molecules. The nucleobases are preferably selected from naturally occurring bases (that is, adenine, guanine, cytosine, thymine and uracil) as well as nucleobase analogues, such as 2,6-diaminopurine, hypoxanthine, 5-methylcytosine, pseudouracil, 5-propynyluracil, and 5-propynylcytosine, which enable a specific binding to the target RNA.

A strong and sequence-specific interaction (that is, a more stable hybrid between the RNA substrate and the oligomer) between the RNA substrate and elements Z_1 and Z_4 is preferred. For this purpose, it is preferred that the following nucleobase analogues be used in oligomeric

sequences of elements Z_1 and Z_4 in place of the standard nucleobases: 2,6-diaminopurine instead of adenine; thymine or 5-propynyluracil instead of uracil; and 5-methylcytosine or 5-propynylcytosine instead of cytosine. 2-Amino-2'-O-alkyladenosines are also preferred (Lamm *et al.*, *Nucleic Acids Res.* 19:3193-3198 (1991)). Furthermore, aromatic systems can be linked to positions 4 and 5 of uracil to produce nucleobase analogues such as phenoxazine, which can improve the stability of the double-strand (Lin *et al.*, *J. Am. Chem. Soc.* 117:3873-3874 (1995)).

Preferred RNA substrates for cleavage by the disclosed compositions have the structure



where Z_1' and Z_4' interact with Z_1 and Z_4 , respectively, where $C^{16.1}$ is cytidine, and where X^{17} is adenosine, guanosine, cytidine, or uridine. Cleavage occurs 3' of X^{17} . Preferably, X^{17} is adenosine, cytidine, or uridine, more preferably X^{17} is adenosine or cytidine, and most preferably X^{17} is adenosine. Preferably, $X^{16.2}$ (that is, the 3' nucleoside in Z_4') is adenosine or guanosine. The target sites in substrates which can be cleaved by the disclosed compositions are distinct from target sites for previous hammerhead ribozymes since previous hammerhead ribozymes require a uridine in position 16.1 of the substrate.

Position $N^{16.2}$, which is the 3' most position present in Z_4' , can be either a guanosine, adenosine, cytidine, or uridine. It is preferred that $N^{16.2}$ is either a guanosine, adenosine, or cytidine. It is more preferred that $N^{16.2}$ is either guanosine or adenosine. It is most preferred that $N^{16.2}$ is guanosine. Preferred substrates for cleavage by the disclosed compounds are those where $N^{16.2}$ is guanosine, adenosine, or cytidine and X^{17} is adenosine. More preferred substrates for cleavage by the disclosed compounds are those where $N^{16.2}$ is guanosine or adenosine and X^{17} is adenosine. Most preferred substrates for cleavage by the disclosed compounds are those where $N^{16.2}$ is guanosine and X^{17} is adenosine.

Flanking elements Z_1 and Z_4 preferably contain, independently of each other, from 3 to 40, and more preferably from 5 to 10, nucleotides or

nucleotide analogues. It is preferred that Z_1 and Z_1' interact to form a stem of at least three base pairs, and that Z_4 and Z_4' interact to form a stem of at least three base pairs. It is more preferred that these stems are adjacent to Z_2 and Z_3 , respectively. It is most preferable that Z_1 and Z_1' interact to form a stem of more than three base pairs, and that Z_4 and Z_4' interact to form a stem of more than three base pairs.

Preferred RNA substrates are those that have little inhibitory secondary structure associated with the target region of the RNA. There are a number of ways to determine which regions of an RNA molecule contain secondary structure, and would therefore be less preferred, and which regions of an RNA molecule have little secondary structure, and therefore, would be more preferred. Preferred methods for determining regions of single-stranded RNA are those that map the single-stranded regions of RNA by selectively reacting with or recognizing these regions. There are many chemicals (dimethyl sulfate (DMS), diethylpyrocarbonate (DEPC), cathoxal (CMCT), carbodiimides) which react with nitrogens at the Watson-Crick face of nucleotides. Nucleotides involved in Watson-Crick base pairing show less reactivity with these chemicals than nucleotides which are not. Enzymatic reactions (using reverse transcriptase, RNase T1, cobra venom nuclease, nuclease S1, nuclease V1) with chemically modified RNA create shortened oligonucleotides whose length is dependant on the base where the chemical reaction occurred. Since chemical reactions occur preferentially at the single stranded regions of the RNA, these techniques indicate where the secondary structure of the RNA is. For example, methods such as dimethyl sulfate and reverse transcription mapping indicate regions of double-stranded RNA. Reverse transcriptase under the appropriate conditions is unable to process through regions of double-stranded RNA, and therefore, there are abortive transcripts which when analyzed by polyacrylamide gel electrophoresis (PAGE) indicate where in the RNA regions of strong secondary structure exist. Examples of these methods are described by Kumar *et al.*, *Biochemistry* 33(2):583-592 (1994), Mandiyan and Boublik, *Nucleic Acids Res.* 18(23):7055-7062 (1990), Bernal and Garcia-Arenal, *RNA* 3(9):1052-

1067 (1997), and Ehresmann *et al.*, *Nucleic Acids Res.* 15(22):9109-9128 (1987).

Another method which assesses which regions of RNA are single-stranded is RNase H mapping. In this method, short, random DNA
5 oligonucleotides are synthesized and mixed with the target RNA. Regions of easy accessibility are hybridized with the short DNA molecules. The DNA-RNA hybrid regions are then cleaved by RNase H. The labeled RNA molecule can then be analyzed by PAGE. By comparing the cleaved molecules with a sequencing ladder, the regions of single-stranded DNA can
10 be inferred. Examples of this method are described by Ho *et al.*, *Nature Biotechnology* 16:59-63 (1998), and in U.S. Patent No. 5,525,468 to McSwiggen.

In vitro selection experiments (Szostak, *TIBS* 19:89-93 (1992)) can also be performed to determine the accessible single-stranded regions of
15 RNA. For example, the target RNA can be mixed with random DNA oligonucleotides that contain primer binding regions which can be used for PCR amplification. Amplification and reselection in an iterative manner will allow for enrichment of those DNA sequences which are capable of binding the RNA. This identifies the regions of the RNA which are accessible for
20 oligonucleotide hybridization. The selection or enrichment step can be any size selection or double-stranded nucleic acid separation technique. For example, Sephadex column chromatography will separate the large, bound DNA:RNA complexes and the small unbound DNA molecules, or nitrocellulose filtration will retain the bound RNA while the unbound DNA
25 molecules will flow through.

There are also a number of methods for optimizing the oligomers for a given substrate. For example, position N' of the oligomers, which has no specific sequence requirements, can be changed to help minimize the possibility of unwanted secondary structure in the oligomers designed for a
30 given target sequence. Also specific base modifications, such as 7-deaza-guanosine or 7-deaza-adenosine, can be utilized in regions having a number of guanines or adenosines to prevent unwanted purine:purine interactions.

Similar substitutions can be accomplished by introducing inosine into guanosine rich regions, that may be present for example in regions Z¹ or Z⁴ of the catalytic oligomer.

Catalytic Core

5 Elements Z₂ and Z₃ are considered to form the catalytic core of the disclosed compositions, and are preferably made up of nucleotide analogues and a small number of ribonucleotides. In elements Z₂ and Z₃ it is preferred that each W (in structure (I)) is C₁-C₃ straight chain or branched alkyl, C₂-C₃ straight chain or branched alkenyl, C₂-C₃ straight chain or branched alkynyl, C₁-C₃ straight chain or branched alkoxy, C₂-C₃ straight chain or branched alkenyloxy, and C₂-C₃ straight chain or branched C₂-C₃ alkynyloxy. It is also preferred that in each X³, X⁴, X⁷ and X¹², W is NH₂, OH-substituted C₁-C₄ alkyl, OH-substituted C₂-C₄ alkenyl, OH-substituted C₁-C₄ alkoxy or OH-substituted C₂-C₄ alkenyloxy. It is more preferred that in each X³, X⁴, X⁷ and X¹², W is NH₂, methoxy, 2-hydroxyethoxy, allyloxy or allyl. It is also preferred that in X¹², W is -H or -OH. It is also preferred that in each X¹³ and X¹⁴, W is C₁-C₄ alkyl, C₂-C₄ alkenyl, C₁-C₄ alkoxy, C₂-C₄ alkenyloxy, OH-substituted C₁-C₄ alkyl, OH-substituted C₂-C₄ alkenyl, OH-substituted C₁-C₄ alkoxy, or OH-substituted C₂-C₄ alkenyloxy. It is more preferred that in each X¹³ and X¹⁴, W is methoxy, 2-hydroxyethoxy or allyloxy.

The subunits in elements Z₂ and Z₃ are preferably nucleotide analogues which can only hybridize weakly with ribonucleotides. Examples of such subunits are nucleotide analogues that contain a substituted or unsubstituted alkyl, alkenyl, alkynyl, alkoxy, alkenyloxy or alkynyloxy group, with preferably 1 to 5 carbon atoms, at the 2' position of ribose. Preferred nucleobases which can be used in elements Z₂ and Z₃ for this purpose are adenin-9-yl, purin-9-yl, uracil-1-yl, cytosin-1-yl, guanin-9-yl and hypoxanthin-9-yl.

The following nucleotides and nucleotide analogues are preferred for element Z₂ (referring to components of structure (I)):

Position X³: B = cytosin-1-yl, V = O, W = allyloxy; B = cytosin-1-yl, V = O, W = allyl;

Position X⁴: B = uracil-1-yl, V = O, W = allyloxy; B = uracil-1-yl, V = O, W = allyl; B = uracil-1-yl, V = O, W = OH;

Position X⁵: B = guanine-9-yl, V = O, W = amino; B = guanine-9-yl, V = O, W = OH;

5 Position X⁶: B = adenine-9-yl, V = O, W = H; B = purine-9-yl, V = O, W = OH; B = adenine-9-yl, V = O, W = OH;

Position X⁷: B = uracil-1-yl, V = O, W = allyloxy; B = cytosine-1-yl, V = O, W = allyl;

10 Position X⁸: B = guanine-9-yl, V = O, W = amino; B = hypoxanthine-9-yl, V = O, W = OH; B = guanine-9-yl, V = O, W = OH;

Position X⁹: B = adenine-9-yl, V = O, W = H; B = purine-9-yl, V = O, W = OH; B = adenine-9-yl, V = O, W = allyloxy.

The following nucleotides and nucleotide analogues are preferred for element Z₂ (referring to components of structure (I)):

15 Position X¹²: B = guanine-9-yl, V = O, W = H; B = 7-deazaguanine-9-yl, V = O, W = OH; B = guanine-9-yl, V = O, W = OH;

Position X¹³: B = adenine-9-yl, V = O, W = allyloxy; or B = adenine-9-yl, V = O, W = 2-hydroxyethoxy; B = purine-9-yl, V = O, W = allyloxy;

20 Position X¹⁴: B = adenine-9-yl, V = O, W = allyloxy; B = purine-9-yl, V = O, W = OH; B = adenine-9-yl, V = O, W = 2-hydroxyethoxy; B = purine-9-yl, V = O, W = allyloxy;

Position X^{15.1}: B = hypoxanthine-9-yl or a functional equivalent thereof, V = O, W = OH.

25 Elements Z₂ and Z₃ interact in a way that allows for the formation of a catalytic structure. In preferred compositions Z₂ and Z₃ interact in a way that allows for the formation of a catalytic structure resembling a hammerhead catalytic structure. One way Z₂ and Z₃ can interact to form a catalytic structure is through the interaction of the nucleotides and/or
30 nucleotide analogues making up Z₂ and Z₃. The disclosed compositions have an RNA cleaving activity independent of RNase H. That is, the disclosed compositions are able to cause cleavage of an RNA substrate without

involving RNase H. Although the disclosed compositions may also be capable of promoting cleavage of RNA by RNase H, it is preferred that they do not.

Desired interaction between Z_2 and Z_3 is preferably enhanced by coupling elements to the 3'-end of Z_2 and/or the 5'-end of Z_3 . A single element (referred to herein as Z_4) can be used in this way to covalently couple elements Z_2 and Z_3 . The structure of such a form of the disclosed compositions would be 5'- Z_1 - Z_2 - Z_4 - Z_3 -3'. Separate elements (referred to herein as Z_4 and Z_7) can be coupled to Z_2 and Z_3 which preferably interact (non-covalently) to stabilize or otherwise enhance the interaction of elements Z_2 and Z_3 . Component (a) of a composition of this form would have the structure 5'- Z_1 - Z_2 - Z_4 -3', and component (b) would have the structure 5'- Z_7 - Z_3 - Z_4 -3'.

It is preferred that elements Z_3 , Z_4 and Z_7 are oligomeric sequences, non-nucleotide linkers, or a combination of oligomeric sequences and non-nucleotide linkers. It is more preferred that elements Z_3 , Z_4 and Z_7 are oligomeric sequences. It is most preferred that these oligomeric sequences interact to form an intramolecular stem (in the case of Z_3) or an intermolecular stem (in the case of Z_4 and Z_7). Such stems preferably contain from 2 to 30 base pairs, and are preferably continuous (that is, lacking unpaired bases). Elements Z_3 , Z_4 and Z_7 preferably are comprised of nucleotides, nucleotide analogues, or both, or are oligonucleotide analogues. It is preferred that Z_3 interacts with itself in such a way as to stabilize the interactions between Z_2 and Z_3 . Similarly, it is preferred that Z_4 interact with Z_7 in such a way as to stabilize the interactions between Z_2 and Z_3 . It is preferred that elements are oligomeric sequences made up of nucleotides and/or nucleotide analogues, oligonucleotide analogues, or a combination, which are able to hybridize with each other.

Element Z_4 can serve as a covalent linker coupling the 3' end of Z_2 to the 5' end of Z_3 . Element Z_4 is preferably made up of either non-nucleotide molecules such as polyethylene glycol, or oligomeric sequences, including nucleotides, nucleotide analogues and oligonucleotide analogues, or a

combination of nucleotides, nucleotide analogues and oligonucleotide analogues. A preferred form of element Z_3 is made up of nucleotides, nucleotide analogues, oligonucleotide analogues, or a combination of nucleotides and nucleotide analogues which are able to interact

5 intramolecularly to form a stem-loop structure. A preferred stem-loop structure for element Z_3 is one containing from 2 to 30 base pairs.

A preferred embodiment of the disclosed compositions is 5'- Z_1 - Z_2 - Z_3 - Z_4 -3' where all of the nucleotides are either 2'-O-allyl-ribonucleotides or 2'-O-methyl-ribonucleotides except for positions X^4 , X^5 , X^6 , X^8 , X^{12} , $X^{13.1}$ which are ribonucleotides unmodified at the 2' position ($W = OH$).

10 Another preferred embodiment of the disclosed compositions is 5'- Z_1 - Z_2 - Z_3 - Z_4 -3' where all of the nucleotides are either 2'-O-allyl-ribonucleotides or 2'-O-methyl-ribonucleotides except for positions X^5 , X^6 , X^8 , X^{12} , $X^{13.1}$ which are ribonucleotides unmodified at the 2' position ($W = OH$).

15 Another preferred embodiment of the disclosed compositions is a composition made up of components (a) and (b) as described above where component (a) is 5'- Z_1 - Z_2 - Z_4 -3' and component (b) is 5'- Z_7 - Z_3 - Z_4 -3', where all of the nucleotides are either 2'-O-allyl-ribonucleotides or 2'-O-methyl-ribonucleotides except for positions X^4 , X^5 , X^6 , X^8 , X^{12} , $X^{13.1}$ which are ribonucleotides unmodified at the 2' position ($W = OH$).

20 Another preferred embodiment of the disclosed compositions is a composition made up of components (a) and (b) as described above where component (a) is 5'- Z_1 - Z_2 - Z_6 -3' and component (b) is 5'- Z_7 - Z_3 - Z_4 -3', where all of the nucleotides are either 2'-O-allyl-ribonucleotides or 2'-O-methyl-ribonucleotides except for positions X^5 , X^6 , X^8 , X^{12} , $X^{13.1}$ which are ribonucleotides unmodified at the 2' position ($W = OH$).

25 A preferred form of the disclosed composition is one in which there is a G added to the 3' end of Z_2 . Taira and co-workers (Amontov and Taira, *J. Am. Chem. Soc.* 118:1624-1628 (1996)) have shown that the stacking energy gained from a guanosine juxtaposed to R^9 of a hammerhead-like ribozyme

stabilizes the formation of a catalytic structure. Thus, it is preferred that the 5' nucleotide of Z_4 is G.

The 3' end of the disclosed compositions can be protected against degradation by exonucleases by, for example, using a nucleotide analogue that is modified at the 3' position of the ribose sugar (for example, by including a substituted or unsubstituted alkyl, alkoxy, alkenyl, alkenyloxy, alkynyl or alkynyloxy group as defined above). The disclosed compositions can also be stabilized against degradation at the 3' end by exonucleases by including a 3'-3'-linked dinucleotide structure (Ortigao *et al.*, *Antisense Research and Development* 2:129-146 (1992)) and/or two modified phospho bonds, such as two phosphorothioate bonds.

The disclosed compositions can also be linked to a prosthetic group in order to improve their cellular uptake and/or to enable a specific cellular localization. Examples of such prosthetic groups are polyamino acids (for example, polylysine), lipids, hormones or peptides. These prosthetic groups are usually linked via the 3' or 5' end of the oligomer either directly or by means of suitable linkers (for example, linkers based on 6-aminohexanol or 6-mercaptohexanol). These linkers are commercially available and techniques suitable for linking prosthetic groups to the oligomer are known to a person skilled in the art.

Increasing the rate of hybridization can be important for the biological activity of the disclosed compositions since in this way it is possible to achieve a higher activity at low concentrations of the composition. This is important for short-lived RNA substrates or RNA substrates that occur less often. A substantial acceleration of the hybridization can be achieved by, for example, coupling positively charged peptides (containing, for example, several lysine residues) to the end of an oligonucleotide (Corey *J. Am. Chem. Soc.* 117:9373-9374 (1995)). The disclosed compositions can be simply modified in this manner using the linkers described above. Alternatively, the rate of hybridization can also be increased by incorporation of subunits which contain sperminyl residues (Schmid and Behr, *Tetrahedron Lett.* 36:1447-

1450 (1995)). Such modifications of the disclosed compositions also improve the ability to bind to RNA substrates having secondary structures.

Synthesis of Oligomers

The disclosed compositions can be synthesized using any suitable method. Many synthesis methods are known. The following techniques are preferred for synthesis of the disclosed compositions. 2'-O-Allyl modified oligomers that contain residual purine ribonucleotides, and bearing a suitable 3'-terminus such as an inverted thymidine residue (Ortigao et al., *Antisense Research and Development* 2:129-146 (1992)) or two phosphorothioate linkages at the 3'-terminus to prevent eventual degradation by 3'-exonucleases, can be synthesized by solid phase β -cyanoethyl phosphoramidite chemistry (Sinha et al., *Nucleic Acids Res.* 12:4539-4557 (1984)) on any commercially available DNA/RNA synthesizer. A preferred method is the 2'-O-*tert*-butyldimethylsilyl (TBDMS) protection strategy for the ribonucleotides (Usman et al., *J. Am. Chem. Soc.* 109:7845-7854 (1987)), and all the required 3'-O-phosphoramidites are commercially available. In addition, the use of aminomethylpolystyrene is preferred as the support material due to its advantageous properties (McCollum and Andrus *Tetrahedron Letters* 32:4069-4072 (1991)). Fluorescein can be added to the 5'-end of a substrate RNA during the synthesis by using commercially available fluorescein phosphoramidites. In general, a desired oligomer can be synthesized using a standard RNA cycle. Upon completion of the assembly, all base labile protecting groups are removed by an 8 hour treatment at 55°C with concentrated aqueous ammonia/ethanol (3:1 v/v) in a sealed vial. The ethanol suppresses premature removal of the 2'-O-TBDMS groups which would otherwise lead to appreciable strand cleavage at the resulting ribonucleotide positions under the basic conditions of the deprotection (Usman et al., *J. Am. Chem. Soc.* 109:7845-7854 (1987)). After lyophilization the TBDMS protected oligomer is treated with a mixture of triethylamine trihydrofluoride/triethylamine/N-methylpyrrolidinone for 2 hours at 60°C to afford fast and efficient removal of the silyl protecting groups under neutral conditions (Wincott et al., *Nucleic Acids Res.* 23:2677-

2684 (1995)). The fully deprotected oligomer can then be precipitated with butanol according to the procedure of Cathala and Brunel (*Nucleic Acids Res.* 18:201 (1990)). Purification can be performed either by denaturing polyacrylamide gel electrophoresis or by a combination of ion-exchange HPLC (Sproat *et al.*, *Nucleosides and Nucleotides* 14:255-273 (1995)) and reversed phase HPLC. For use in cells, it is preferred that synthesized oligomers be converted to their sodium salts by precipitation with sodium perchlorate in acetone. Traces of residual salts are then preferably removed using small disposable gel filtration columns that are commercially available. As a final step it is preferred that the authenticity of the isolated oligomers is checked by matrix assisted laser desorption mass spectrometry (Picles *et al.*, *Nucleic Acids Res.* 21:3191-3196 (1993)) and by nucleoside base composition analysis. In addition, a functional cleavage test with the oligomer on the corresponding chemically synthesized short oligoribonucleotide substrate is also preferred.

Cleavage of RNA Substrates

The disclosed compositions have a very high *in vivo* activity since the RNA cleavage is promoted by protein factors that are present in the nucleus or cytoplasm of the cell. Examples of such protein factors which can increase the activity of hammerhead ribozymes are, for example, the nucleocapsid protein NCp7 of HIV1 (Müller *et al.*, *J. Mol. Biol.* 242:422-429 (1994)) and the heterogeneous nuclear ribonucleoprotein A1 (Heidenreich *et al.*, *Nucleic Acids Res.* 23:2223-2228 (1995)). Thus, long RNA transcripts can be cleaved efficiently within the cell by the disclosed compositions.

The disclosed compositions can be used in pharmaceutical compositions that contain one or several oligomers as the active substance, and, optionally, pharmaceutically acceptable auxiliary substances, additives and carriers. Such pharmaceutical compositions are suitable for the production of an agent to specifically inactivate the expression of genes in eukaryotes, prokaryotes and viruses, especially of human genes such as tumor genes or viral genes or RNA molecules in a cell. Further areas of application are the inactivation of the expression of plant genes or insect genes. Thus, the

disclosed compositions can be used as drugs for humans and animals as well as a pesticide for plants.

A variety of methods are available for delivering the disclosed compositions to cells. For example, in general, the disclosed compositions can be incorporated within or on microparticles. As used herein, microparticles include liposomes, virosomes, microspheres and microcapsules formed of synthetic and/or natural polymers. Methods for making microcapsules and microspheres are known to those skilled in the art and include solvent evaporation, solvent casting, spray drying and solvent extension. Examples of useful polymers which can be incorporated into various microparticles include polysaccharides, polyanhydrides, polyorthoesters, polyhydroxides and proteins and peptides.

Liposomes can be produced by standard methods such as those reported by Kim *et al.*, *Biochim. Biophys. Acta*, 728:339-348 (1983); Liu *et al.*, *Biochim. Biophys. Acta*, 1104:95-101 (1992); and Lee *et al.*, *Biochim. Biophys. Acta*, 1103:185-197 (1992); Wang *et al.*, *Biochem.*, 28:9508-9514 (1989)). Such methods have been used to deliver nucleic acid molecules to the nucleus and cytoplasm of cells of the MOLT-3 leukemia cell line (Thierry and Dritschilo, *Nucl. Acids Res.*, 20:5691-5698 (1992)). Alternatively, the disclosed compositions can be incorporated within microparticles, or bound to the outside of the microparticles, either ionically or covalently.

Cationic liposomes or microcapsules are microparticles that are particularly useful for delivering negatively charged compounds such as the disclosed compounds, which can bind ionically to the positively charged outer surface of these liposomes. Various cationic liposomes have previously been shown to be very effective at delivering nucleic acids or nucleic acid-protein complexes to cells both *in vitro* and *in vivo*, as reported by Felgner *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987); Felgner, *Advanced Drug Delivery Reviews*, 5:163-187 (1990); Clarenc *et al.*, *Anti-Cancer Drug Design*, 8:81-94 (1993). Cationic liposomes or microcapsules can be prepared using mixtures including one or more lipids containing a cationic side group in a sufficient quantity such that the liposomes or microcapsules

formed from the mixture possess a net positive charge which will ionically bind negatively charged compounds. Examples of positively charged lipids that may be used to produce cationic liposomes include the aminolipid dioleoyl phosphatidyl ethanolamine (PE), which possesses a positively charged primary amino head group; phosphatidylcholine (PC), which possess positively charged head groups that are not primary amines; and N[1-(2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium ("DOTMA," see Felgner *et al.*, *Proc. Natl. Acad. Sci USA*, 84:7413-7417 (1987); Felgner *et al.*, *Nature*, 337:387-388 (1989); Felgner, *Advanced Drug Delivery Reviews*, 5:163-187 (1990)).

A preferred form of microparticle for delivery of the disclosed compositions are heme-bearing microparticles. In these microparticles, heme is intercalated into or covalently conjugated to the outer surface of the microparticles. Heme-bearing microparticles offer an advantage in that since they are preferentially bound and taken up by cells that express the heme receptor, such as hepatocytes, the amount of drug required for an effective dose is significantly reduced. Such targeted delivery may also reduce systemic side effects that can arise from using relatively high drug concentrations in non-targeted delivery methods. Preferred lipids for forming heme-bearing microparticles are 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) and dioleoyl phosphatidyl ethanolamine (DOPE). The production and use of heme-bearing microparticles are described in PCT application WO 95/27480 by Innovir.

The disclosed compositions can also be encapsulated by or coated on cationic liposomes which can be injected intravenously into a mammal. This system has been used to introduce DNA into the cells of multiple tissues of adult mice, including endothelium and bone marrow, where hematopoietic cells reside (see, for example, Zhu *et al.*, *Science*, 261:209-211 (1993)).

Liposomes containing the disclosed compositions can be administered systemically, for example, by intravenous or intraperitoneal administration, in an amount effective for delivery of the disclosed compositions to targeted cells. Other possible routes include trans-dermal or oral, when used in

conjunction with appropriate microparticles. Generally, the total amount of the liposome-associated oligomer administered to an individual will be less than the amount of the unassociated oligomer that must be administered for the same desired or intended effect.

5 Compositions including various polymers such as the polylactic acid and polyglycolic acid copolymers, polyethylene, and polyorthoesters and the disclosed compositions can be delivered locally to the appropriate cells by using a catheter or syringe. Other means of delivering such compositions locally to cells include using infusion pumps (for example, from Alza
10 Corporation, Palo Alto, California) or incorporating the compositions into polymeric implants (see, for example, Johnson and Lloyd-Jones, eds., *Drug Delivery Systems* (Chichester, England: Ellis Horwood Ltd., 1987), which can effect a sustained release of the therapeutic compositions to the immediate area of the implant.

15 For therapeutic applications the active substance is preferably administered at a concentration of 0.01 to 10,000 $\mu\text{g/kg}$ body weight, more preferably of 0.1 to 1000 $\mu\text{g/kg}$ body weight. The administration can, for example, be carried out by injection, inhalation (for example as an aerosol), as a spray, orally (for example as tablets, capsules, coated tablets etc.),
20 topically or rectally (for example as suppositories).

 The disclosed compositions can be used in a method for the specific inactivation of the expression of genes in which an active concentration of the composition is taken up into a cell so that the composition specifically cleaves a predetermined RNA molecule which is present in the cell, the cleavage
25 preferably occurring catalytically. Similar compositions, which are described in U.S. Patent No. 5,334,711, have been used successfully in mice to inactivate a gene (Lyngstadaas *et al.*, *EMBO J.* 14:5224-5229 (1995)). This process can be carried out *in vitro* on cell cultures as well as *in vivo* on living organisms (prokaryotes or eukaryotes such as humans, animals or plants).

30 The disclosed compositions can also be used as RNA restriction enzymes to cleave RNA molecules (in, for example, cell free *in vitro* reactions). The disclosed compositions can also be used in a reagent kit for

the restriction cleavage of RNA molecules which contains, for example, an oligomer and suitable buffer substances. In this case the oligomer and the buffer substances can be present in the form of solutions, suspensions or solids such as powders or lyophilisates. The reagents can be present
5 together, separated from one another or optionally also on a suitable carrier. The disclosed compositions can also be used as a diagnostic agent or to identify the function of unknown genes.

The present invention will be further understood by reference to the following non-limiting examples.

10

Examples

Example 1: Cleavage reactions which indicate that an inosine substitution at position 15.1 can effectively cleave $N^{16,2}C^{14,1}H^{17}$.

A set of 12 substrates was synthesized which covered each
15 permutation of the $N^{16,2}C^{14,1}H^{17}$ motif where H^{17} is not guanosine. The oligomers and the corresponding substrates used in the cleavage assays are shown in Table 1. Each of the substrates was labeled with fluorescein at the 5' end and an inverted thymidine cap was used on the 3'-end. A set of four catalytic oligomers was synthesized, providing an appropriately matched
20 catalytic oligomer for each of the substrates. Each of these catalytic oligomers had an inosine at position 15.1. A control substrate and catalytic oligomer were also synthesized in which there was a U at position 16.1 of the substrate and an A at position 15.1 of the catalytic oligomer.

Table 1

$N^{16.2}N^{16.1}H^{17}$ Triplet		Substrate sequence	
5	ACC	FI-GAAUACCGGUCGC*T	(SEQ ID NO:4)
	ACA	FI-GAAUACAGGUCGC*T	(SEQ ID NO:5)
	ACU	FI-GAAUACUGGUCGC*T	(SEQ ID NO:6)
10	GCC	FI-GAAUGCCGGUCGC*T	(SEQ ID NO:7)
	GCA	FI-GAAUGCAGGUCGC*T	(SEQ ID NO:8)
	GCU	FI-GAAUGCUGGUCGC*T	(SEQ ID NO:9)
15	CCC	FI-GAAUCCCGGUCGC*T	(SEQ ID NO:10)
	CCA	FI-GAAUCCAGGUCGC*T	(SEQ ID NO:11)
	CCU	FI-GAAUCCUGGUCGC*T	(SEQ ID NO:12)
20	UCC	FI-GAAUCCCGGUCGC*T	(SEQ ID NO:13)
	UCA	FI-GAAUUCAGGUCGC*T	(SEQ ID NO:14)
	UCU	FI-GAAUUCUGGUCGC*T	(SEQ ID NO:15)
	GUC	FI-GAAUGUCGGUCGC*T	(SEQ ID NO:16)
Targeted triplet		Catalytic oligomer sequence	
25	ACH	gcgaccuGAuGaggccgugaggccGaaIuauc*T	(SEQ ID NO:17)
	GCH	gcgaccuGAuGaggccgugaggccGaaIcauc*T	(SEQ ID NO:18)
	CCH	gcgaccuGAuGaggccgugaggccGaaIgauc*T	(SEQ ID NO:19)
	UCH	gcgaccuGAuGaggccgugaggccGaaIaauc*T	(SEQ ID NO:20)
30	GUC	gcgaccuGAuGaggccgugaggccGaaAcauc*T	(SEQ ID NO:21)
FI = Fluorescein label *T = 3'-3' inverted thymidine A, C, G, I, U = ribonucleotides (I is inosine) a, c, g, u = 2'-O-allyl-ribonucleotides			

The above substrates and catalytic oligomers were used in cleavage reactions to determine the ability of an inosine at position 15.1 to overcome the requirement of a U at position 16.1 for cleavage. All of the reactions were performed using the following protocol. The reactions were typically done in 100 μ l and they contained distilled, autoclaved H_2O , 10 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4, 5 μ M ribozyme, and 0.25 μ M substrate. The catalytic oligomer, substrate, and buffer were added together and heated to

95°C for 5 minutes. After cooling to room temperature over 5 minutes the reactions were brought to 10 mM MgCl₂, mixed, and placed at 37°C. 10 μ L aliquots were removed at specific time intervals (10, 30, 60, and 120 minutes) and added to 3 μ L of loading buffer (95% formamide, 100 mM EDTA pH 8.0, 0.05% bromophenol blue) to quench the reaction. Samples were analyzed by 20% polyacrylamide gel electrophoresis. Gels were analyzed on a Molecular Dynamics Fluorescence Imager. The results of cleavage reactions of this type, using the substrates and catalytic oligomers shown in Table 1, are shown in Table 2.

10

Table 2

	N ^{14,2} N ^{14,1} H ¹⁷ Triplet	After mixing	10	30	60	120
15	I ^{13,1} U ^{13,2} Catalytic oligomer					
	ACC	4.4	28.2	58.1	91.5	91.5
	ACA	7.7	71.8	84.7	93.1	94.8
	ACU	1.8			58.7	70.5
20	I ^{13,1} C ^{13,2} Catalytic oligomer					
	GCC	1.62	39.6	59.9	82.0	87.0
	GCA	13.7	65.3	78.7	89.7	93.1
25	GCU	—			64.3	74.8
	I ^{13,1} G ^{13,2} Catalytic oligomer					
	CCC	—			34.33	45.38
30	CCA	1.1	18.8	45.5	70.8	80.63
	CCU	2.0			28.4	36.7
	I ^{13,1} A ^{13,2} Catalytic oligomer					
35	UCC	6.8			57.0	64.7
	UCA	1.6			39.6	60.8
	UCU	3.3			41.1	53.1
	A ^{13,1} C ^{13,2} Catalytic oligomer					
40	GUC	1.6	38.5	66.5	93.5	

The numbers represent the percentage of substrate cleaved at the indicated time point (which were at 0, 10, 30, 60, and 120 minutes after starting the reaction). The results indicate that substrates with a C at position 16.1 are able to be cleaved by catalytic oligomers containing an I at position 15.1. While there are differences between the various substrates at the 120 minute time point, the data show that a substrate with a C at position 16.1 in conjunction with a catalytic oligomer with an I at position 15.1 is able to effectively cleave in all backgrounds, indicating that the substitution of an I at position 15.1 does in fact allow for the cleavage of any appropriate substrate containing a N^{16.2}C^{16.1}H¹⁷ site.

Initial rates of cleavage of the twelve substrates having C^{16.1}, and the control substrate having U^{16.1}, by the corresponding catalytic oligomers (all shown in Table 1) were determined using single turnover kinetics. Single turnover kinetics were assessed by mixing 2.5 μ l of a 100 μ M ribozyme solution, 2.5 μ l of a 10 μ M solution of 5' fluorescein labeled substrate, and 10 μ l of a 100 mM Tris-HCl pH 7.4 solution. The mixture was diluted to a final volume of 90 μ l, heated to 95°C for 5 minutes, and cooled to 37°C. The reaction was started by adding 10 μ l of a 100 mM MgCl₂ solution. The final concentrations of the reaction components were 250 nM substrate, 2.5 μ M ribozyme, and 10 mM MgCl₂. Ten microliter samples were removed at various times and mixed with 10 μ l of a 100 mM EDTA, bromphenol blue solution to stop the reaction. Cleavage products were separated from unreacted substrate by PAGE and were quantitated on a Molecular Dynamics Fluorescence Imager.

The data, measured in fraction of substrate cleaved versus time, were fitted to the equation:

$$\text{frac}[P] = H_0(1 - e^{-k_1 t})/S_0$$

as described by Jankowsky and Schwenzler, *Nucl. Acids Res.* 24:433 (1996).

The calculated values of k_1 for the various ribozymes are shown in Table 3.

Table 3

$N^{16,2}N^{16,1}H^{17}$			
Triplet			
5	gcgacccuGAuGaggccgugaggccGaaIuauc*T (SEQ ID NO:17)		
		k_2 (min ⁻¹)	Substrate sequence
	ACC	0.07	FI-GAAUACCGGUCGC*T (SEQ ID NO:4)
	ACA	0.36	FI-GAAUACAGGUCGC*T (SEQ ID NO:5)
10	gcgacccuGAuGaggccgugaggccGaaIcauc*T (SEQ ID NO:18)		
		k_2 (min ⁻¹)	Substrate sequence
	GCC	0.12	FI-GAAUGCCGGUCGC*T (SEQ ID NO:7)
	GCA	0.48	FI-GAAUGCAGGUCGC*T (SEQ ID NO:8)
15	gcgacccuGAuGaggccgugaggccGaaIgauc*T (SEQ ID NO:19)		
		k_2 (min ⁻¹)	Substrate sequence
	GCU	0.05	FI-GAAUGCUGGUCGC*T (SEQ ID NO:9)
20	gcgacccuGAuGaggccgugaggccGaaIaauuc*T (SEQ ID NO:20)		
		k_2 (min ⁻¹)	Substrate sequence
	CCC	<0.01	FI-GAAUCCCGGUCGC*T (SEQ ID NO:10)
	CCA	0.04	FI-GAAUCCAGGUCGC*T (SEQ ID NO:11)
25	gcgacccuGAuGaggccgugaggccGaaIaauc*T (SEQ ID NO:21)		
		k_2 (min ⁻¹)	Substrate sequence
	CCU	<0.01	FI-GAAUCCUGGUCGC*T (SEQ ID NO:12)
30	gcgacccuGAuGaggccgugaggccGaaAcauc*T (SEQ ID NO:21)		
		k_2 (min ⁻¹)	Substrate sequence
	UCC	<0.01	FI-GAAUCCCGGUCGC*T (SEQ ID NO:13)
	UCA	<0.01	FI-GAAUUCAGGUCGC*T (SEQ ID NO:14)
35	gcgacccuGAuGaggccgugaggccGaaAcauc*T (SEQ ID NO:21)		
	GUC	0.13	FI-GAAUGUCGGUCGC*T (SEQ ID NO:16)
40	FI = Fluorescein label		
	*T = 3'-3' inverted thymidine		
	A, C, G, I, U = ribonucleotides (I is inosine)		
	a, c, g, u = 2'-O-allyl-ribonucleotides		

The results show that substrates with $A^{16,2}C^{16,1}H^{17}$ and $G^{16,2}C^{16,1}H^{17}$ triplets are cleaved at a high rate. Comparison to the control catalytic oligomer having an A at position 15.1 (to cleave a substrate with a

G^{16.2}U^{16.1}C¹⁷ triplet) shows that substrates with A^{16.2}C^{16.1}A¹⁷ and G^{16.2}C^{16.1}A¹⁷ triplets (to be cleaved by a catalytic oligomer with an I at position 15.1) have an initial rate of cleavage that is higher than the corresponding control reactions involving reactants with a standard A^{15.1}-U^{16.1} base pair.

5 Example 2: Rate of cleavage at GCH and ACH triplets by catalytic oligomers containing only ribonucleotides

Cleavage of substrate RNA by all-ribonucleotide versions of oligomers (no modifications) designed to cleave GCH and ACH triplets was assessed. The oligomers used were SEQ ID NO:17 (cleaves after ACH triplets) (Table 1) and SEQ ID NO:18 (cleaves after GCH triplets) (Table 1). The
 10 corresponding short fluorescent labelled substrates, SEQ ID NOS:4, 5, and 6 (containing ACH triplets) (Table 1) and SEQ ID NOS:7, 8, and 9 (containing GCH triplets) (Table 1) were used with catalytic oligomers SEQ ID NO:17 and SEQ ID NO:18 respectively.

15 The reactions were performed under single-turnover kinetic conditions, using 2.5 μ M catalytic oligomer and 250 nM substrate, both at pH 6.0 in the presence of 10 mM Mg²⁺ and also at pH 7.4 in the presence of 1 mM Mg²⁺. All other reaction conditions were as in Example 1.

The data were analyzed as in Example 1. The k_2 values for the 6
 20 combinations of all-ribonucleotide catalytic oligomer and corresponding substrates are shown in Table 4.

		Table 4	
		k_2 (min) ⁻¹	
		pH 6.0; 10 mM Mg ²⁺	pH 7.4; 1 mM Mg ²⁺
25	Triplet		
	GCA	0.39	2.32
	GCC	0.18	2.03
	GCU	0.03	0.10
30	ACA	0.41	1.12
	ACC	0.17	0.71
	ACU	0.02	0.06

The all ribonucleotide oligomers targeting GCA, GCC, ACA, and ACC sites
 35 have a higher rate of cleavage than the all ribonucleotide oligomers targeting GCU or ACU sites. Furthermore, Table 4 indicates the cleavage activity is

Ribozyme
L-Cheng-mod.

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(54) Title: HAMMERHEAD RIBOZYMES WITH EXTENDED CLEAVAGE RULE			
<p>Diagram illustrating the structure of a Hammerhead ribozyme with extended cleavage rule. The structure shows various nucleotides (1-18) and their interactions, including STEM LOOP II, CORE, and STEM LOOP I.</p>			
(57) Abstract			
<p>Disclosed are compositions having an RNA-cleavage activity, as well as their use for cleaving RNA-substrates <i>in vitro</i> and <i>in vivo</i>. The compositions contain an active center, the subunits of which are selected from nucleotides and/or nucleotide analogues, as well as flanking regions contributing to the formation of a specific hybridization with an RNA substrate. Preferred compositions form, in combination with an RNA substrate, a structure resembling a hammerhead structure. The active center of the disclosed compositions is characterized by the presence of 1^{15.1} which allows cleavage of RNA substrates having C^{14.1}.</p>			

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HAMMERHEAD RIBOZYMES WITH EXTENDED CLEAVAGE RULE

Background of the Invention

The present invention is in the field of compositions having RNA-cleavage activity.

5 Hammerhead ribozymes are an example of catalytic RNA molecules which are able to recognize and cleave a given specific RNA substrate (Hotchins *et al.*, *Nucleic Acids Res.* 14:3627 (1986); Keese and Symons, in *Viroids and viroid-like pathogens* (J.J. Semanchik, publ., CRC-Press, Boca Raton, Florida, 1987), pages 1-47). The catalytic center of hammerhead
10 ribozymes is flanked by three stems and can be formed by adjacent sequence regions of the RNA or also by regions which are separated from one another by many nucleotides. Figure 1 shows a diagram of such a catalytically active hammerhead structure. The stems have been denoted I, II and III. The nucleotides are numbered according to the standard nomenclature for
15 hammerhead ribozymes (Hertel *et al.*, *Nucleic Acids Res.* 20:3252 (1992)). In this nomenclature, bases are denoted by a number which relates their position relative to the 5' side of the cleavage site. Furthermore, each base that is involved in a stem or loop region has an additional designation (which is denoted by a decimal point and then another number) that defines the
20 position of that base within the stem or loop. A designation of N^{11.3} would indicate that this base is involved in a paired region and that it is the third base in that stem going away from the core region. This accepted convention for describing hammerhead derived ribozymes allows for the nucleotides involved in the core of the enzyme to always have the same
25 number relative to all of the other nucleotides. The size of the stems involved in substrate binding or core formation can be any size and of any sequence, and the position of A⁹, for example, will remain the same relative to all of the other core nucleotides. Nucleotides designated, for example, N^{^12} or N^{^9^} represent an inserted nucleotide where the position of the caret
30 (^) relative to the number denotes whether the insertion is before or after the indicated nucleotide. Thus, N^{^12} represents a nucleotide inserted before

nucleotide position 12, and N^{9a} represents a nucleotide inserted after nucleotide position 9.

The consensus sequence of the catalytic core structure is described by Ruffner and Uhlenbeck (*Nucleic Acids Res.* 18:6025-6029 (1990)). Perriman
5 *et al.* (*Gene* 113:157-163 (1992)) have meanwhile shown that this structure can also contain variations, for example, naturally occurring nucleotide insertions such as N^{9a} and N^{12} . Thus, the positive strand of the satellite RNA of the tobacco ring-spot virus does not contain any of the two nucleotide insertions while the +RNA strand of the virusoid of the lucerne
10 transient streak virus (vLTSV) contains a $N^{9a} = U$ insertion which can be mutated to C or G without loss of activity (Sheldon and Symons, *Nucleic Acids Res.* 17:5679-5685 (1989)). Furthermore, in this special case, $N^7 = A$ and $R^{15.1} = A$. On the other hand, the minus strand of the carnation stunt associated viroid (- CarSV) is quite unusual since it contains both nucleotide
15 insertions, that is $N^{12} = A$ and $N^{9a} = C$ (Hernandez *et al.*, *Nucleic Acids Res.* 20:6323-6329 (1992)). In this viroid $N^7 = A$ and $R^{15.1} = A$. In addition, this special hammerhead structure exhibits a very effective self-catalytic cleavage despite the more open central stem.

Possible uses of hammerhead ribozymes include, for example,
20 generation of RNA restriction enzymes and the specific inactivation of the expression of genes in, for example, animal, human or plant cells and prokaryotes, yeasts and plasmodia. A particular biomedical interest is based on the fact that many diseases, including many forms of tumors, are related to the overexpression of specific genes. Inactivating such genes by cleaving
25 the associated mRNA represents a possible way to control and eventually treat such diseases. Moreover there is a great need to develop antiviral, antibacterial, and antifungal pharmaceutical agents. Ribozymes have potential as such anti-infective agents since RNA molecules vital to the survival of the organism can be selectively destroyed.

30 One of the greatest impediments to using hammerhead based ribozymes for pharmaceutical agents is the limited availability of acceptable targets in normal Gerlach designs and when targeting pre-formed half

hammerhead structures (see WO 97/18312). In addition to needing the correct hybridizing sequences for substrate binding, substrates for hammerhead ribozymes have been shown to strongly prefer the triplet $N^{16.2}U^{16.1}H^{17}$ where N can be any nucleotide, U is uridine, and H is either adenosine, cytidine, or uridine (Koizumi *et al.*, *FEBS Lett.* 228, 228-230 (1988); Ruffner *et al.*, *Biochemistry* 29, 10695-10702 (1990); Perriman *et al.*, *Gene* 113, 157-163 (1992)). The fact that changes to this general rule for substrate specificity result in non-functional substrates implies that there are "non core compatible" structures which are formed when substrates are provided which deviate from the stated requirements. Evidence along these lines was recently reported by Uhlenbeck and co-workers (*Biochemistry* 36:1108-1114 (1997)) when they demonstrated that the substitution of a G at position 17 caused a functionally catastrophic base pair between G¹⁷ and C³ to form, both preventing the correct orientation of the scissile bond for cleavage and the needed tertiary interactions of C³ (Murray *et al.*, *Biochem. J.* 311:487-494 (1995)). The strong preference for a U at position 16.1 may exist for similar reasons. Many experiments have been done in an attempt to isolate ribozymes which are able to efficiently relieve the requirement of a U at position 16.1, however, attempts to find hammerhead type ribozymes which can cleave substrates having a base other than a U at position 16.1 have proven impossible (Perriman *et al.*, *Gene* 113, 157-163 (1992)).

Efficient catalytic molecules with reduced or altered requirements in the cleavage region are highly desirable because their isolation would greatly increase the number of available target sequences that molecules of this type could cleave. For example, it would be desirable to have a ribozyme variant that could efficiently cleave substrates containing triplets other than $N^{16.2}U^{16.1}H^{17}$ since this would increase the number of potential target cleavage sites.

Chemically modified oligonucleotides which contain a block of deoxyribonucleotides in the middle region of the molecule have potential as pharmaceutical agents for the specific inactivation of the expression of genes (Giles *et al.*, *Nucleic Acids Res.* 20:763-770 (1992)). These oligonucleotides

can form a hybrid DNA-RNA duplex in which the DNA bound RNA strand is degraded by RNase H. Such oligonucleotides are considered to promote cleavage of the RNA and so cannot be characterized as having an RNA-cleaving activity nor as cleaving an RNA molecule (the RNase H is
5 cleaving). A significant disadvantage of these oligonucleotides for *in vivo* applications is their low specificity, since hybrid formation, and thus cleavage, can also take place at undesired positions on the RNA molecules.

Previous attempts to recombinantly express catalytically active RNA molecules in the cell by transfecting the cell with an appropriate gene have
10 not proven to be very effective since a very high expression was necessary to inactivate specific RNA substrates. In addition the vector systems which are available now cannot generally be applied. Furthermore, unmodified ribozymes cannot be administered directly due to the sensitivity of RNA to degradation by RNases and their interactions with proteins. Thus, chemically
15 modified active substances have to be used in order to administer hammerhead ribozymes exogenously (discussed, for example, by Heidenreich *et al.*, *J. Biol. Chem.* 269:2131-2138 (1994); Kichntopf *et al.*, *EMBO J.* 13:4645-4652 (1994); Paoletta *et al.*, *EMBO J.* 11:1913-1919 (1992); and Usman *et al.*, *Nucleic Acids Symp. Ser.* 31:163-164 (1994)).

20 U.S. Pat. No. 5,334,711 describes such chemically modified active substances based on synthetic catalytic oligonucleotide structures with a length of 35 to 40 nucleotides which are suitable for cleaving a nucleic acid target sequence and contain modified nucleotides that contain an optionally substituted alkyl, alkenyl or alkynyl group with 1 - 10 carbon atoms at the
25 2'-O atom of the ribose. These oligonucleotides contain modified nucleotide building blocks and form a structure resembling a hammerhead structure. These oligonucleotides are able to cleave specific RNA substrates.

The use of a large number of deoxyribonucleotides in the hybridization arms or in the active center can lead to a loss of specificity due
30 to an activation of RNase H since sequences which are related to the desired target sequence can also be cleaved. Moreover, catalytic DNA oligomers are

not particularly well suited for *in vivo* applications due to interactions with proteins, and lack of resistance to degradation by nucleases.

It is therefore an object of the present invention to provide compositions that cleave RNA, and in particular to provide RNA-cleaving oligomers which at the same time have a high stability, activity, and specificity.

It is another object of the present invention to provide compositions that cleave RNA substrates having a cleavage site triplet other than $N^{16.2}U^{16.1}H^{17}$.

10

Summary of the Invention

Disclosed are compositions having an RNA-cleavage activity, as well as their use for cleaving RNA substrates *in vitro* and *in vivo*. The compositions contain an active center, the subunits of which are selected from nucleotides and/or nucleotide analogues, as well as flanking regions contributing to the formation of a specific hybridization with an RNA substrate. Preferred compositions form, in combination with an RNA substrate, a structure resembling a hammerhead structure. The active center of the disclosed compositions is characterized by the presence of $I^{15.1}$ which allows cleavage of RNA substrates having $C^{16.1}$.

15

Brief Description of the Drawings

Figure 1 is a diagram of a hammerhead structure and the corresponding nomenclature (SEQ ID NO:1). Cleavage occurs between H^{17} and $N^{1.1}$ to generate the 2',3'-cyclic phosphate at H^{17} .

Figure 2 is a diagram of an RNA substrate (SEQ ID NO:3) in association with an example of an oligomer (SEQ ID NO:2) that cleaves the RNA substrate. The structure formed by the oligomer and the substrate resembles the structure of a hammerhead ribozyme. In this case, the substrate makes up half of stems I and III, and loops I and III are not present. Cleavage occurs 3' of H^{17} .

20

Figure 3 is a diagram showing the interaction of the $A^{15.1}-U^{16.1}$ base pair in hammerhead ribozymes (top), and the predicted isostructural

interaction of a I^{15.1}-C^{16.1} base pair (bottom) that replaces the A^{15.1}-U^{16.1} base pair.

Figure 4A is a graph of fraction of cleavage product versus time (in minutes) for the cleavage of a short 5'-fluorescein labelled oligoribonucleotide substrate (SEQ ID NO:8) containing a GCA site by four variants of 2'-O-allylated 5-ribo catalytic oligomers each containing a different nucleobase at position N⁷ (U, C, A and G; SEQ ID NOS:18, 22, 23, and 24, respectively).

Figure 4B is a graph of fraction of cleavage product versus time (in minutes) for the cleavage of a short 5'-fluorescein labelled oligoribonucleotide substrate of SEQ ID NO:8 containing a GCA site by four variants of 2'-O-allylated 5-ribo catalytic oligomers each containing a different nucleobase or base analogue at position N⁷ (U, 5-nitroindole, I, and quinazoline-2, 4-dione; SEQ ID NOS:18, 27, 25, and 26, respectively).

Detailed Description of the Invention

Disclosed are compositions having an RNA-cleavage activity, as well as their use for cleaving RNA-substrates *in vitro* and *in vivo*. The compositions contain an active center, the subunits of which are selected from nucleotides and/or nucleotide analogues, as well as flanking regions contributing to the formation of a specific hybridization with an RNA substrate. Preferred compositions form, in combination with an RNA substrate, a structure resembling a hammerhead structure. The active center of the disclosed compositions is characterized by the presence of I^{15.1} which allows cleavage of RNA substrates having C^{16.1}.

All naturally occurring hammerhead ribozymes have an A^{15.1}-U^{16.1} base pair. In addition, it is known that substrates for ribozymes based on the consensus hammerhead sequence strongly prefer a substrate that contains an N^{16.2}U^{16.1}H¹⁷ triplet in which H¹⁷ is not a guanosine (Koizumi *et al.*, *FEBS Lett.* 228, 228-230 (1988); Ruffner *et al.*, *Biochemistry* 29, 10695-10702 (1990); Perriman *et al.*, *Gene* 113, 157-163 (1992)). Many experiments have been done in an attempt to isolate ribozymes which are able to efficiently relieve the requirement of a U at position 16.1, however, attempts to find

ribozymes which can cleave substrates having a base other than a U at position 16.1 have proven impossible (Perriman *et al.*, *Gene* 113, 157-163 1992, Singh *et al.*, *Antisense and Nucleic Acid Drug Development* 6:165-168 (1996)).

5 However, examination of the recently published X-ray crystal structures (Pley *et al.*, *Nature* 372:68-74 (1994), Scott *et al.*, *Cell* 81:991-1002 (1995), and Scott *et al.*, *Science* 274:2065-2069 (1996)) led to the realization that the A^{15.1}-U^{16.1} interaction is a non-standard base pair with a single hydrogen bond between the exocyclic amine (N6) of the adenosine and
10 the 4-oxo group of the uridine. Modeling studies (based on the crystal structure) then led to the discovery that the interaction of the wild-type A^{15.1}-U^{16.1} base pair can be spatially mimicked by replacement with an I^{15.1}-C^{16.1} base pair that adopts an isostructural orientation and which preserves the required contact of the 2-keto group of C^{16.1} with A⁶ of the uridine turn. In
15 the model, the polarity of the stabilizing hydrogen bond between positions 15.1 and 16.1 is reversed in the I^{15.1}-C^{16.1} interaction, but the correct orientation of the bases around this bond is maintained.

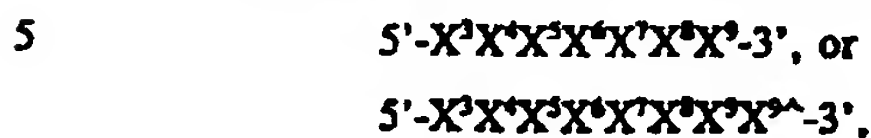
 It has been discovered that Gerlach type ribozyme analogues containing an inosine at position 15.1 readily cleave RNA substrates
20 containing an N^{16.2}C^{16.1}H¹⁷ triplet. Based on this, disclosed are compositions, preferably synthetic oligomers, which cleave a nucleic acid target sequence containing the triplet N^{16.2}C^{16.1}H¹⁷. It is preferred that H¹⁷ is not guanosine. The ability to cleave substrates having N^{16.2}C^{16.1}X¹⁷ triplets effectively doubles the number of targets available for cleavage by compositions of the type
25 disclosed.

Compositions Having an RNA-cleavage Activity

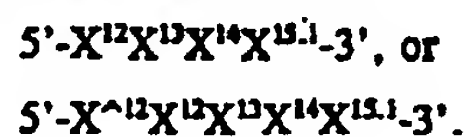
 Specifically disclosed is a composition that cleaves an RNA substrate, where the composition includes components (a) and (b), where component (a) includes a structure 5'-Z₁-Z₂-3' and component (b) includes a structure
30 5'-Z₃-Z₄-3'. Components (a) and (b) can either be separate molecules or can be covalently coupled. Elements Z₁ and Z₄ in components (a) and (b) are each oligomeric sequences which are made up of nucleotides, nucleotide

analogues, or a combination of both, or are oligonucleotide analogues. The oligomeric sequences of elements Z_1 and Z_4 specifically interact with the RNA substrate, preferably by hybridization.

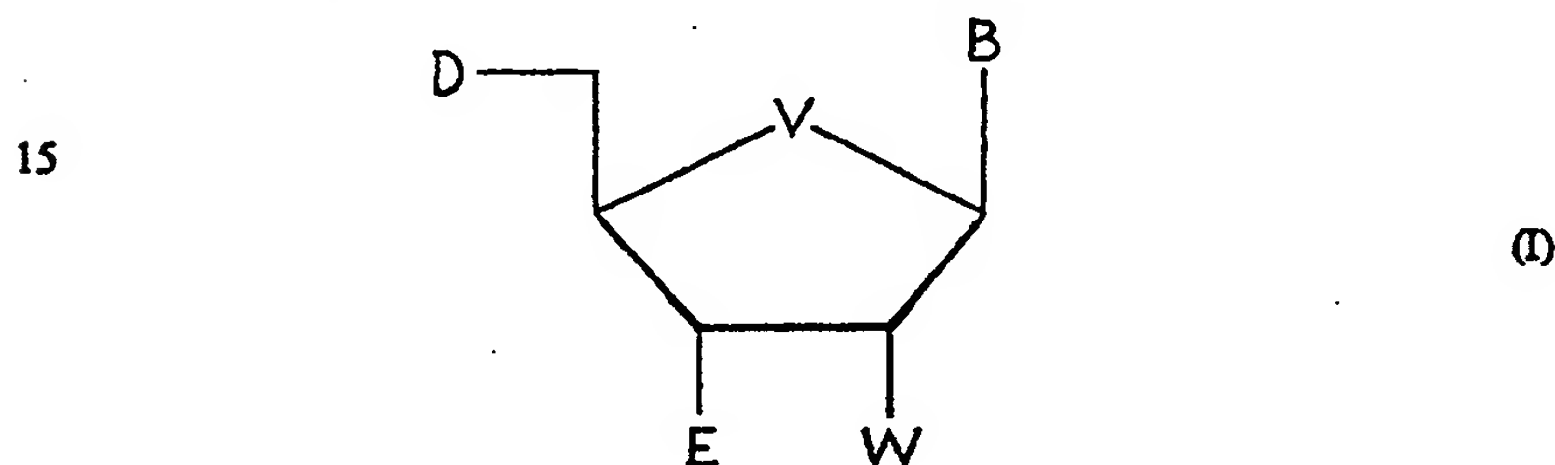
In these preferred compositions, element Z_1 has a structure of



and element Z_2 has a structure of



10 Elements Z_2 and Z_3 in these preferred compositions are made up of nucleotides, nucleotide analogues, or a combination of both. The nucleotides and nucleotide analogues in elements Z_2 and Z_3 each have the structure



20 In structure (I) each B can be adenin-9-yl, cytosin-1-yl, guanin-9-yl, uracil-1-yl, uracil-5-yl, hypoxanthin-9-yl, thymine-1-yl, 5-methylcytosin-1-yl, 2,6-diaminopurin-9-yl, purin-9-yl, 7-deazaadenin-9-yl, 7-deazaguanin-9-yl, 5-propynylcytosin-1-yl, 5-propynyluracil-1-yl, isoguanin-9-yl, 2-aminopurin-9-yl, 6-methyluracil-1-yl, 4-thiouracil-1-yl, 2-pyrimidone-1-yl, quinazoline-2,4-
 25 dione-1-yl, xanthin-9-yl, N²-dimethylguanin-9-yl or a functional equivalent thereof;

Each V can be an O, S, NH, or CH₂ group.

Each W can be -H, -OH, -COOH, -CONH₂, -CONHR¹, -CONR¹R², -NH₂, -NHR¹, -NR¹R², -NHCOR¹, -SH, SR¹, -F, -ONH₂, -ONHR¹, -
 30 ONR¹R², -NHOH, -NHOR¹, -NR²OH, -NR²OR¹, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkyl, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyl, substituted or unsubstituted C₂-C₁₀ straight

chain or branched alkynyl, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkoxy, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyloxy, and substituted or unsubstituted C₂-C₁₀ straight chain or branched alkynyloxy. The substituents for W groups are independently
 5 halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto. R¹ and R² can be substituted or unsubstituted alkyl, alkenyl, or alkynyl groups, where the substituents are independently halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto.

D and E are residues which together form a phosphodiester or
 10 phosphorothioate diester bond between adjacent nucleosides or nucleoside analogues or together form an analogue of an internucleosidic bond.

B is hypoxanthin-9-yl, or a functional equivalent thereof, in X^{15.1}; B can be guanine-9-yl, hypoxanthin-9-yl or 7-deazaguanine-9-yl in X³, X⁴, and X¹²; B can be adenine-9-yl, 2,6-diaminopurine-9-yl, purine-9-yl or 7-deazadenine-9-yl in X⁶, X⁹, X¹³, and X¹⁴; B can be uracil-1-yl, uracil-5-yl, thymine-1-yl or 5-propynyluracil-1-yl in X⁴; B can be cytosine-1-yl, 5-methylcytosine-1-yl or 5-propynylcytosine-1-yl in X³; and B can be adenine-9-yl, cytosine-1-yl, guanine-9-yl, uracil-1-yl, uracil-5-yl, hypoxanthin-9-yl, thymine-1-yl, 5-methylcytosine-1-yl, 2,6-diaminopurine-9-yl, purine-9-yl, 7-deazaadenine-9-yl, 7-deazaguanine-9-yl, 5-propynylcytosine-1-yl, 5-propynyluracil-1-yl, isoguanine-9-yl, 2-aminopurine-9-yl, 6-methyluracil-1-yl, 4-thiouracil-1-yl, 2-pyrimidone-1-yl, quinazoline-2,4-dione-1-yl, xanthine-9-yl, N²-dimethylguanine-9-yl, or a functional equivalent thereof in X⁷, X⁸, and X¹². B of X^{15.1} is preferably hypoxanthin-9-yl or an analog where no hydrogen bond can form between
 15 any group at the 2 position of the base and the 2-oxo group of C^{16.1}.
 20 Preferably, B is not guanine-9-yl in X^{15.1}.

B in X³, X⁴, X⁵, X⁶, X⁸, X⁹, X¹², X¹³, and X¹⁴ can also be a functionally equivalent nucleobase within the context of the catalytic core of a hammerhead ribozyme. For example, C³, U⁴, G⁵, and A⁶ in hammerhead
 30 ribozymes form a structure closely resembling a uridine turn in a tRNA (Pley *et al.*, *Nature* 372:68-74 1994). Other groups of nucleotides can also form uridine turns and so nucleotides X³, X⁴, X⁵, X⁶ may be replaced as a group

with nucleotides or nucleotide analogues that have the potential to form a structure resembling a uridine turn. Similarly, the sheared base pairs in the catalytic core of hammerhead ribozymes have interactions that may be similar to interactions of other non-canonical base pairs. Knowledge of the crystal
5 structure of the catalytic core of hammerhead ribozymes, combined with the discovery that a Gerlach type hammerhead ribozyme in which a non-canonical base pair has been replaced with an isostructural non-canonical base pair is active, indicates that analogous isostructural base pair replacements should be possible elsewhere in the catalytic core.

10 **Definitions**

As used herein, oligomer refers to oligomeric molecules composed of subunits where the subunits can be of the same class (such as nucleotides) or a mixture of classes (such as nucleotides and ethylene glycol). It is preferred that the disclosed oligomers be oligomeric sequences, non-nucleotide linkers,
15 or a combination of oligomeric sequences and non-nucleotide linkers. It is more preferred that the disclosed oligomers be oligomeric sequences. Oligomeric sequences are oligomeric molecules where each of the subunits includes a nucleobase (that is, the base portion of a nucleotide or nucleotide analogue) which can interact with other oligomeric sequences in a base-
20 specific manner. The hybridization of nucleic acid strands is a preferred example of such base-specific interactions. Oligomeric sequences preferably are comprised of nucleotides, nucleotide analogues, or both, or are oligonucleotide analogues.

Non-nucleotide linkers can be any molecule, which is not an
25 oligomeric sequence, that can be covalently coupled to an oligomeric sequence. Preferred non-nucleotide linkers are oligomeric molecules formed of non-nucleotide subunits. Examples of such non-nucleotide linkers are described by Letsinger and Wu, (*J. Am. Chem. Soc.* 117:7323-7328 (1995)), Benseler *et al.*, (*J. Am. Chem. Soc.* 115:8483-8484 (1993)) and Fu *et al.*,
30 (*J. Am. Chem. Soc.* 116:4591-4598 (1994)). Preferred non-nucleotide linkers, or subunits for non-nucleotide linkers, include substituted or unsubstituted C₁-C₁₀ straight chain or branched alkyl, substituted or

unsubstituted C₂-C₁₀ straight chain or branched alkenyl, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkynyl, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkoxy, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyloxy, and substituted or
5 unsubstituted C₂-C₁₀ straight chain or branched alkynyloxy. The substituents for these preferred non-nucleotide linkers (or subunits) can be halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto.

As used herein, nucleoside refers to adenosine, guanosine, cytidine, uridine, 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-
10 deoxycytidine, or thymidine. A nucleoside analogue is a chemically modified form of nucleoside containing a chemical modification at any position on the base or sugar portion of the nucleoside. As used herein, the term nucleoside analogue encompasses, for example, both nucleoside analogues based on naturally occurring modified nucleosides, such as inosine and pseudouridine,
15 and nucleoside analogues having other modifications, such as modifications to the 2' position of the sugar. As used herein, nucleotide refers to a phosphate derivative of nucleosides as described above, and a nucleotide analogue is a phosphate derivative of nucleoside analogues as described above. The subunits of oligonucleotide analogues, such as peptide nucleic acids, are also
20 considered to be nucleotide analogues.

As used herein, a ribonucleotide is a nucleotide having a 2' hydroxyl function. Analogously, a 2'-deoxyribonucleotide is a nucleotide having only 2' hydrogens. Thus, ribonucleotides and deoxyribonucleotides as used herein refer to naturally occurring nucleotides having nucleoside components
25 adenosine, guanosine, cytidine, and uridine, or 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine, and thymidine, respectively, without any chemical modification. Ribonucleosides, deoxyribonucleosides, ribonucleoside analogues and deoxyribonucleoside analogues are similarly defined except that they lack the phosphate group, or an analogue of the
30 phosphate group, found in nucleotides and nucleotide analogues.

As used herein, oligonucleotide analogues are polymers of nucleic acid-like material with nucleic acid-like properties, such as sequence

dependent hybridization, that contain at one or more positions, a modification away from a standard RNA or DNA nucleotide. A preferred example of an oligonucleotide analogue is peptide nucleic acid.

As used herein, base pair refers to a pair of nucleotides or nucleotide analogues which interact through one or more hydrogen bonds. The term
5 base pair is not limited to interactions generally characterized as Watson-Crick base pairs, but includes non-canonical or sheared base pair interactions (Topal and Fresco, *Nature* 263:285 (1976); Lomant and Fresco, *Prog. Nucl. Acid Res. Mol. Biol.* 15:185 (1975)). Thus, nucleotides A^{13,1} and U^{14,1} form
10 a base pair in hammerhead ribozymes (see Figure 1) but the base pair is non-canonical (see Figure 3).

The internucleosidic linkage between two nucleosides can be achieved by phosphodiester bonds or by modified phospho bonds such as by phosphorothioate groups or other bonds such as, for example, those described
15 in U.S. Pat. No. 5,334,711.

Flanking Elements Z₁ and Z₄

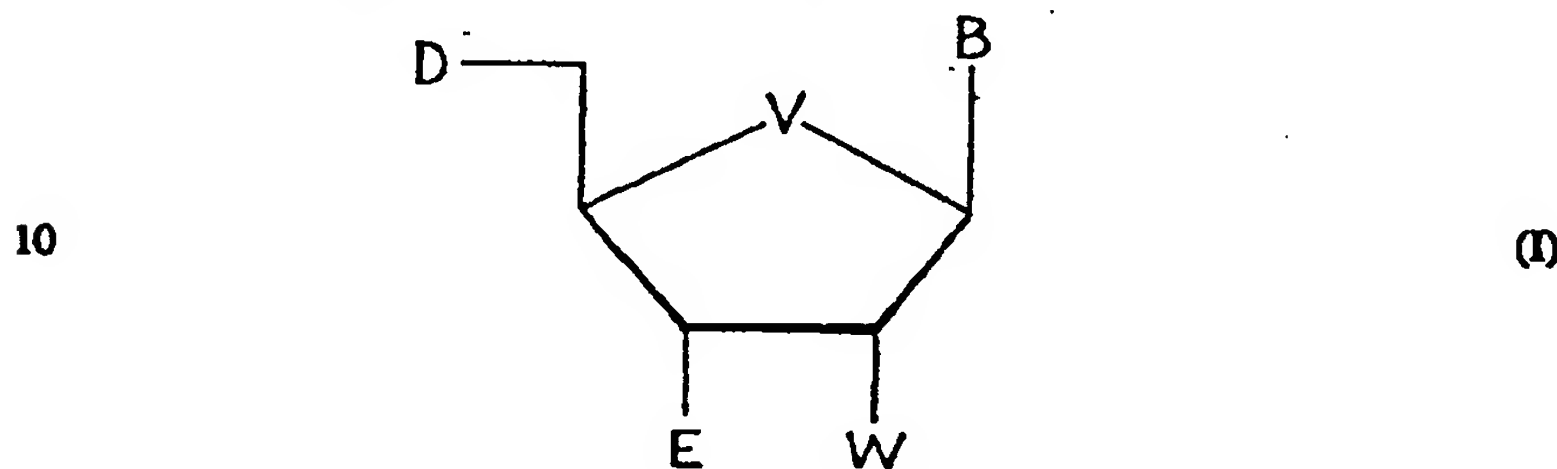
The monomeric subunits of elements Z₁ and Z₄ which flank the active center (formed by elements Z₂ and Z₃) are preferably nucleotides and/or nucleotide analogues. Elements Z₁ and Z₄ are designed so that they
20 specifically interact, preferably by hybridization, with a given RNA substrate and, together with the active center Z₂ and Z₃, form a structure (preferably a structure resembling that of a hammerhead ribozyme) which specifically cleaves the RNA substrate.

The subunits of elements Z₁ and Z₄ can, on the one hand, be
25 ribonucleotides. However, it is preferred that the number of ribonucleotides be as small as possible since the presence of ribonucleotides reduces the *in vivo* stability of the oligomers. Elements Z₁ and Z₄ (and also the active center Z₂ and Z₃) preferably do not contain any ribonucleotides at the positions containing pyrimidine nucleobases. Such positions preferably
30 contain nucleotide analogues.

The use of a large number of deoxyribonucleotides in elements Z₁ and Z₄ is also less preferred since undesired interactions with proteins can occur

or an unintended RNase H-sensitive DNA-RNA hybrid could form. Thus, elements Z_1 and Z_4 each preferably contain (1) no ribonucleotides, and (2) no sequences of more than 3 consecutive deoxyribonucleotides.

The subunits of elements Z_1 and Z_4 are preferably nucleotides, nucleotide analogues, or a combination. Preferably, the nucleotides and nucleotide analogues in elements Z_1 and Z_4 each have the structure



In structure (I) each B can be adenin-9-yl, cytosin-1-yl, guanin-9-yl, uracil-1-yl, uracil-5-yl, hypoxanthin-9-yl, thymine-1-yl, 5-methylcytosin-1-yl, 2,6-diaminopurin-9-yl, purin-9-yl, 7-deazaadenin-9-yl, 7-deazaguanin-9-yl, 5-propynylcytosin-1-yl, 5-propynyluracil-1-yl, isoguanin-9-yl, 2-aminopurin-9-yl, 6-methyluracil-1-yl, 4-thiouracil-1-yl, 2-pyrimidone-1-yl, quinazoline-2,4-dione-1-yl, xanthin-9-yl, N^2 -dimethylguanin-9-yl or a functional equivalent thereof;

Each V can be an O, S, NH, or CH_2 group.

Each W can be -H, -OH, -COOH, -CONH₂, -CONHR¹, -CONR¹R², -NH₂, -NHR¹, -NR¹R², -NHCOR¹, -SH, SR¹, -F, -ONH₂, -ONHR¹, -ONR¹R², -NHOH, -NHOR¹, -NR²OH, -NR²OR¹, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkyl, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyl, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkynyl, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkoxy, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyloxy, and substituted or unsubstituted C₂-C₁₀ straight chain or branched alkynyloxy. The substituents for W groups are independently halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto. R¹ and R² can be substituted or unsubstituted alkyl, alkenyl, or

alkynyl groups, where the substituents are independently halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto.

D and E are residues which together form a phosphodiester or phosphorothioate diester bond between adjacent nucleosides or nucleoside analogues or together form an analogue of an internucleosidic bond.

For elements Z_1 and Z_4 having nucleotide and/or nucleotide analogues of structure (I), it is preferred that each W is substituted or unsubstituted C_1 - C_{10} straight chain or branched alkoxy, C_2 - C_{10} straight chain or branched alkenyloxy, or C_2 - C_{10} straight chain or branched alkynyloxy.

In addition, the flanking elements Z_1 and Z_4 can also contain nucleotide analogues such as peptide nucleic acids (also referred to as peptidic nucleic acids; see for example Nielsen *et al.*, *Science* 254:1497-1500 (1991), and Dueholm *et al.*, *J. Org. Chem.* 59:5767-5773 (1994)). In this case the coupling of individual subunits can, for example, be achieved by acid amide bonds. Elements Z_1 and Z_4 , when based on peptide nucleic acids, can be coupled to elements Z_2 and Z_3 , based on nucleotides or nucleotide analogues, using either suitable linkers (see, for example, Petersen *et al.*, *BioMed. Chem. Lett.* 5:1119-1121 (1995)) or direct coupling (Bergmann *et al.*, *Tetrahedron Lett.* 36:6823-6826 (1995)). Where elements Z_1 and Z_4 contain a combination of nucleotides (and/or nucleotide analogues) and peptide nucleic acid, similar linkages can be used to couple the different parts.

The subunits of the flanking elements Z_1 and Z_4 contain nucleobases or nucleobase analogues which can hybridize or interact with nucleobases that occur naturally in RNA molecules. The nucleobases are preferably selected from naturally occurring bases (that is, adenine, guanine, cytosine, thymine and uracil) as well as nucleobase analogues, such as 2,6-diaminopurine, hypoxanthine, 5-methylcytosine, pseudouracil, 5-propynyluracil, and 5-propynylcytosine, which enable a specific binding to the target RNA.

A strong and sequence-specific interaction (that is, a more stable hybrid between the RNA substrate and the oligomer) between the RNA substrate and elements Z_1 and Z_4 is preferred. For this purpose, it is preferred that the following nucleobase analogues be used in oligomeric

sequences of elements Z_1 and Z_4 in place of the standard nucleobases: 2,6-diaminopurine instead of adenine; thymine or 5-propynyluracil instead of uracil; and 5-methylcytosine or 5-propynylcytosine instead of cytosine. 2-Amino-2'-O-alkyladenosines are also preferred (Lamm *et al.*, *Nucleic Acids Res.* 19:3193-3198 (1991)). Furthermore, aromatic systems can be linked to positions 4 and 5 of uracil to produce nucleobase analogues such as phenoxazine, which can improve the stability of the double-strand (Lin *et al.*, *J. Am. Chem. Soc.* 117:3873-3874 (1995)).

Preferred RNA substrates for cleavage by the disclosed compositions have the structure



where Z_1' and Z_4' interact with Z_1 and Z_4 , respectively, where $C^{16.1}$ is cytidine, and where X^{17} is adenosine, guanosine, cytidine, or uridine. Cleavage occurs 3' of X^{17} . Preferably, X^{17} is adenosine, cytidine, or uridine, more preferably X^{17} is adenosine or cytidine, and most preferably X^{17} is adenosine. Preferably, $X^{16.2}$ (that is, the 3' nucleoside in Z_4') is adenosine or guanosine. The target sites in substrates which can be cleaved by the disclosed compositions are distinct from target sites for previous hammerhead ribozymes since previous hammerhead ribozymes require a uridine in position 16.1 of the substrate.

Position $N^{16.2}$, which is the 3' most position present in Z_4' , can be either a guanosine, adenosine, cytidine, or uridine. It is preferred that $N^{16.2}$ is either a guanosine, adenosine, or cytidine. It is more preferred that $N^{16.2}$ is either guanosine or adenosine. It is most preferred that $N^{16.2}$ is guanosine. Preferred substrates for cleavage by the disclosed compounds are those where $N^{16.2}$ is guanosine, adenosine, or cytidine and X^{17} is adenosine. More preferred substrates for cleavage by the disclosed compounds are those where $N^{16.2}$ is guanosine or adenosine and X^{17} is adenosine. Most preferred substrates for cleavage by the disclosed compounds are those where $N^{16.2}$ is guanosine and X^{17} is adenosine.

Flanking elements Z_1 and Z_4 preferably contain, independently of each other, from 3 to 40, and more preferably from 5 to 10, nucleotides or

nucleotide analogues. It is preferred that Z_1 and Z_1' interact to form a stem of at least three base pairs, and that Z_4 and Z_4' interact to form a stem of at least three base pairs. It is more preferred that these stems are adjacent to Z_2 and Z_3 , respectively. It is most preferable that Z_1 and Z_1' interact to form a stem of more than three base pairs, and that Z_4 and Z_4' interact to form a stem of more than three base pairs.

Preferred RNA substrates are those that have little inhibitory secondary structure associated with the target region of the RNA. There are a number of ways to determine which regions of an RNA molecule contain secondary structure, and would therefore be less preferred, and which regions of an RNA molecule have little secondary structure, and therefore, would be more preferred. Preferred methods for determining regions of single-stranded RNA are those that map the single-stranded regions of RNA by selectively reacting with or recognizing these regions. There are many chemicals (dimethyl sulfate (DMS), diethylpyrocarbonate (DEPC), cathoxal (CMCT), carbodiimides) which react with nitrogens at the Watson-Crick face of nucleotides. Nucleotides involved in Watson-Crick base pairing show less reactivity with these chemicals than nucleotides which are not. Enzymatic reactions (using reverse transcriptase, RNase T1, cobra venom nuclease, nuclease S1, nuclease V1) with chemically modified RNA create shortened oligonucleotides whose length is dependant on the base where the chemical reaction occurred. Since chemical reactions occur preferentially at the single stranded regions of the RNA, these techniques indicate where the secondary structure of the RNA is. For example, methods such as dimethyl sulfate and reverse transcription mapping indicate regions of double-stranded RNA. Reverse transcriptase under the appropriate conditions is unable to process through regions of double-stranded RNA, and therefore, there are abortive transcripts which when analyzed by polyacrylamide gel electrophoresis (PAGE) indicate where in the RNA regions of strong secondary structure exist. Examples of these methods are described by Kumar *et al.*, *Biochemistry* 33(2):583-592 (1994), Mandiyan and Boublik, *Nucleic Acids Res.* 18(23):7055-7062 (1990), Bernal and Garcia-Arenal, *RNA* 3(9):1052-

1067 (1997), and Ehresmann *et al.*, *Nucleic Acids Res.* 15(22):9109-9128 (1987).

Another method which assesses which regions of RNA are single-stranded is RNase H mapping. In this method, short, random DNA oligonucleotides are synthesized and mixed with the target RNA. Regions of easy accessibility are hybridized with the short DNA molecules. The DNA-RNA hybrid regions are then cleaved by RNase H. The labeled RNA molecule can then be analyzed by PAGE. By comparing the cleaved molecules with a sequencing ladder, the regions of single-stranded DNA can be inferred. Examples of this method are described by Ho *et al.*, *Nature Biotechnology* 16:59-63 (1998), and in U.S. Patent No. 5,525,468 to McSwiggen.

In vitro selection experiments (Szostak, *TIBS* 19:89-93 (1992)) can also be performed to determine the accessible single-stranded regions of RNA. For example, the target RNA can be mixed with random DNA oligonucleotides that contain primer binding regions which can be used for PCR amplification. Amplification and reselection in an iterative manner will allow for enrichment of those DNA sequences which are capable of binding the RNA. This identifies the regions of the RNA which are accessible for oligonucleotide hybridization. The selection or enrichment step can be any size selection or double-stranded nucleic acid separation technique. For example, Sephadex column chromatography will separate the large, bound DNA:RNA complexes and the small unbound DNA molecules, or nitrocellulose filtration will retain the bound RNA while the unbound DNA molecules will flow through.

There are also a number of methods for optimizing the oligomers for a given substrate. For example, position N' of the oligomers, which has no specific sequence requirements, can be changed to help minimize the possibility of unwanted secondary structure in the oligomers designed for a given target sequence. Also specific base modifications, such as 7-deaza-guanosine or 7-deaza-adenosine, can be utilized in regions having a number of guanosines or adenosines to prevent unwanted purine:purine interactions.

Similar substitutions can be accomplished by introducing inosine into guanosine rich regions, that may be present for example in regions Z^1 or Z^4 of the catalytic oligomer.

Catalytic Core

5 Elements Z_2 and Z_3 are considered to form the catalytic core of the disclosed compositions, and are preferably made up of nucleotide analogues and a small number of ribonucleotides. In elements Z_2 and Z_3 it is preferred that each W (in structure (I)) is C_1 - C_3 straight chain or branched alkyl, C_2 - C_3 straight chain or branched alkenyl, C_2 - C_3 straight chain or branched alkynyl, C_1 - C_3 straight chain or branched alkoxy, C_2 - C_3 straight chain or branched alkenyloxy, and C_2 - C_3 straight chain or branched alkynyloxy. It is also preferred that in each X^3 , X^4 , X^7 and X^{12} , W is NH_2 , OH-substituted C_1 - C_4 alkyl, OH-substituted C_2 - C_4 alkenyl, OH-substituted C_1 - C_4 alkoxy or OH-substituted C_2 - C_4 alkenyloxy. It is more preferred that in each X^3 , X^4 , X^7 and X^{12} , W is NH_2 , methoxy, 2-hydroxyethoxy, allyloxy or allyl. It is also preferred that in X^{12} , W is -H or -OH. It is also preferred that in each X^{13} and X^{14} , W is C_1 - C_4 alkyl, C_2 - C_4 alkenyl, C_1 - C_4 alkoxy, C_2 - C_4 alkenyloxy, OH-substituted C_1 - C_4 alkyl, OH-substituted C_2 - C_4 alkenyl, OH-substituted C_1 - C_4 alkoxy, or OH-substituted C_2 - C_4 alkenyloxy. It is more preferred that in each X^{13} and X^{14} , W is methoxy, 2-hydroxyethoxy or allyloxy.

The subunits in elements Z_2 and Z_3 are preferably nucleotide analogues which can only hybridize weakly with ribonucleotides. Examples of such subunits are nucleotide analogues that contain a substituted or unsubstituted alkyl, alkenyl, alkynyl, alkoxy, alkenyloxy or alkynyloxy group, with preferably 1 to 5 carbon atoms, at the 2' position of ribose. Preferred nucleobases which can be used in elements Z_2 and Z_3 for this purpose are adenin-9-yl, purin-9-yl, uracil-1-yl, cytosin-1-yl, guanin-9-yl and hypoxanthin-9-yl.

The following nucleotides and nucleotide analogues are preferred for element Z_2 (referring to components of structure (I)):

Position X^3 : B = cytosin-1-yl, V = O, W = allyloxy; B = cytosin-1-yl, V = O, W = allyl;

Position X⁴: B = uracil-1-yl, V = O, W = allyloxy; B = uracil-1-yl, V = O, W = allyl; B = uracil-1-yl, V = O, W = OH;

Position X⁵: B = guanine-9-yl, V = O, W = amino; B = guanine-9-yl, V = O, W = OH;

5 Position X⁶: B = adenine-9-yl, V = O, W = H; B = purine-9-yl, V = O, W = OH; B = adenine-9-yl, V = O, W = OH;

Position X⁷: B = uracil-1-yl, V = O, W = allyloxy; B = cytosine-1-yl, V = O, W = allyl;

10 Position X⁸: B = guanine-9-yl, V = O, W = amino; B = hypoxanthine-9-yl, V = O, W = OH; B = guanine-9-yl, V = O, W = OH;

Position X⁹: B = adenine-9-yl, V = O, W = H; B = purine-9-yl, V = O, W = OH; B = adenine-9-yl, V = O, W = allyloxy.

The following nucleotides and nucleotide analogues are preferred for element Z₂ (referring to components of structure (I)):

15 Position X¹²: B = guanine-9-yl, V = O, W = H; B = 7-deazaguanine-9-yl, V = O, W = OH; B = guanine-9-yl, V = O, W = OH;

Position X¹³: B = adenine-9-yl, V = O, W = allyloxy; or B = adenine-9-yl, V = O, W = 2-hydroxyethoxy; B = purine-9-yl, V = O, W = allyloxy;

20 Position X¹⁴: B = adenine-9-yl, V = O, W = allyloxy; B = purine-9-yl, V = O, W = OH; B = adenine-9-yl, V = O, W = 2-hydroxyethoxy; B = purine-9-yl, V = O, W = allyloxy;

Position X^{15.1}: B = hypoxanthine-9-yl or a functional equivalent thereof, V = O, W = OH.

25 Elements Z₂ and Z₃ interact in a way that allows for the formation of a catalytic structure. In preferred compositions Z₂ and Z₃ interact in a way that allows for the formation of a catalytic structure resembling a hammerhead catalytic structure. One way Z₂ and Z₃ can interact to form a catalytic structure is through the interaction of the nucleotides and/or
30 nucleotide analogues making up Z₂ and Z₃. The disclosed compositions have an RNA cleaving activity independent of RNase H. That is, the disclosed compositions are able to cause cleavage of an RNA substrate without

involving RNase H. Although the disclosed compositions may also be capable of promoting cleavage of RNA by RNase H, it is preferred that they do not.

Desired interaction between Z_2 and Z_3 is preferably enhanced by coupling elements to the 3'-end of Z_2 and/or the 5'-end of Z_3 . A single element (referred to herein as Z_4) can be used in this way to covalently couple elements Z_2 and Z_3 . The structure of such a form of the disclosed compositions would be 5'- Z_1 - Z_2 - Z_4 - Z_3 -3'. Separate elements (referred to herein as Z_6 and Z_7) can be coupled to Z_2 and Z_3 which preferably interact (non-covalently) to stabilize or otherwise enhance the interaction of elements Z_2 and Z_3 . Component (a) of a composition of this form would have the structure 5'- Z_1 - Z_2 - Z_6 -3', and component (b) would have the structure 5'- Z_7 - Z_3 -3'.

It is preferred that elements Z_3 , Z_6 and Z_7 are oligomeric sequences, non-nucleotide linkers, or a combination of oligomeric sequences and non-nucleotide linkers. It is more preferred that elements Z_3 , Z_6 and Z_7 are oligomeric sequences. It is most preferred that these oligomeric sequences interact to form an intramolecular stem (in the case of Z_3) or an intermolecular stem (in the case of Z_6 and Z_7). Such stems preferably contain from 2 to 30 base pairs, and are preferably continuous (that is, lacking unpaired bases). Elements Z_3 , Z_6 and Z_7 preferably are comprised of nucleotides, nucleotide analogues, or both, or are oligonucleotide analogues. It is preferred that Z_3 interacts with itself in such a way as to stabilize the interactions between Z_2 and Z_3 . Similarly, it is preferred that Z_6 interact with Z_7 in such a way as to stabilize the interactions between Z_2 and Z_3 . It is preferred that elements are oligomeric sequences made up of nucleotides and/or nucleotide analogues, oligonucleotide analogues, or a combination, which are able to hybridize with each other.

Element Z_4 can serve as a covalent linker coupling the 3' end of Z_2 to the 5' end of Z_3 . Element Z_4 is preferably made up of either non-nucleotide molecules such as polyethylene glycol, or oligomeric sequences, including nucleotides, nucleotide analogues and oligonucleotide analogues, or a

combination of nucleotides, nucleotide analogues and oligonucleotide analogues. A preferred form of element Z_3 is made up of nucleotides, nucleotide analogues, oligonucleotide analogues, or a combination of nucleotides and nucleotide analogues which are able to interact

5 intramolecularly to form a stem-loop structure. A preferred stem-loop structure for element Z_3 is one containing from 2 to 30 base pairs.

A preferred embodiment of the disclosed compositions is $5'-Z_1-Z_2-Z_3-Z_4-3'$ where all of the nucleotides are either 2'-O-allyl-ribonucleotides or 2'-O-methyl-ribonucleotides except for positions X^4 , X^5 , X^6 , X^8 , X^{12} , $X^{15.1}$ which are ribonucleotides unmodified at the 2' position ($W = OH$).

10 Another preferred embodiment of the disclosed compositions is $5'-Z_1-Z_2-Z_3-Z_4-3'$ where all of the nucleotides are either 2'-O-allyl-ribonucleotides or 2'-O-methyl-ribonucleotides except for positions X^5 , X^6 , X^8 , X^{12} , $X^{15.1}$ which are ribonucleotides unmodified at the 2' position ($W = OH$).

15 Another preferred embodiment of the disclosed compositions is a composition made up of components (a) and (b) as described above where component (a) is $5'-Z_1-Z_2-Z_4-3'$ and component (b) is $5'-Z_7-Z_3-Z_4-3'$, where all of the nucleotides are either 2'-O-allyl-ribonucleotides or 2'-O-methyl-ribonucleotides except for positions X^4 , X^5 , X^6 , X^8 , X^{12} , $X^{15.1}$ which are ribonucleotides unmodified at the 2' position ($W = OH$).

20 Another preferred embodiment of the disclosed compositions is a composition made up of components (a) and (b) as described above where component (a) is $5'-Z_1-Z_7-Z_4-3'$ and component (b) is $5'-Z_7-Z_3-Z_4-3'$, where all of the nucleotides are either 2'-O-allyl-ribonucleotides or 2'-O-methyl-ribonucleotides except for positions X^5 , X^6 , X^8 , X^{12} , $X^{15.1}$ which are ribonucleotides unmodified at the 2' position ($W = OH$).

25 A preferred form of the disclosed composition is one in which there is a G added to the 3' end of Z_2 . Taira and co-workers (Amontov and Taira, *J. Am. Chem. Soc.* 118:1624-1628 (1996)) have shown that the stacking energy gained from a guanosine juxtaposed to R^9 of a hammerhead-like ribozyme

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stabilizes the formation of a catalytic structure. Thus, it is preferred that the 5' nucleotide of Z_6 is G.

The 3' end of the disclosed compositions can be protected against degradation by exonucleases by, for example, using a nucleotide analogue that is modified at the 3' position of the ribose sugar (for example, by including a substituted or unsubstituted alkyl, alkoxy, alkenyl, alkenyloxy, alkynyl or alkynyloxy group as defined above). The disclosed compositions can also be stabilized against degradation at the 3' end by exonucleases by including a 3'-3'-linked dinucleotide structure (Ortigao *et al.*, *Antisense Research and Development* 2:129-146 (1992)) and/or two modified phospho bonds, such as two phosphorothioate bonds.

The disclosed compositions can also be linked to a prosthetic group in order to improve their cellular uptake and/or to enable a specific cellular localization. Examples of such prosthetic groups are polyamino acids (for example, polylysine), lipids, hormones or peptides. These prosthetic groups are usually linked via the 3' or 5' end of the oligomer either directly or by means of suitable linkers (for example, linkers based on 6-aminohexanol or 6-mercaptohexanol). These linkers are commercially available and techniques suitable for linking prosthetic groups to the oligomer are known to a person skilled in the art.

Increasing the rate of hybridization can be important for the biological activity of the disclosed compositions since in this way it is possible to achieve a higher activity at low concentrations of the composition. This is important for short-lived RNA substrates or RNA substrates that occur less often. A substantial acceleration of the hybridization can be achieved by, for example, coupling positively charged peptides (containing, for example, several lysine residues) to the end of an oligonucleotide (Corey *J. Am. Chem. Soc.* 117:9373-9374 (1995)). The disclosed compositions can be simply modified in this manner using the linkers described above. Alternatively, the rate of hybridization can also be increased by incorporation of subunits which contain sperminyl residues (Schmid and Behr, *Tetrahedron Lett.* 36:1447-

1450 (1995)). Such modifications of the disclosed compositions also improve the ability to bind to RNA substrates having secondary structures.

Synthesis of Oligomers

The disclosed compositions can be synthesized using any suitable method. Many synthesis methods are known. The following techniques are preferred for synthesis of the disclosed compositions. 2'-O-Allyl modified oligomers that contain residual purine ribonucleotides, and bearing a suitable 3'-terminus such as an inverted thymidine residue (Ortigao et al., *Antisense Research and Development* 2:129-146 (1992)) or two phosphorothioate linkages at the 3'-terminus to prevent eventual degradation by 3'-exonucleases, can be synthesized by solid phase β -cyanoethyl phosphoramidite chemistry (Sinha et al., *Nucleic Acids Res.* 12:4539-4557 (1984)) on any commercially available DNA/RNA synthesizer. A preferred method is the 2'-O-*tert*-butyldimethylsilyl (TBDMS) protection strategy for the ribonucleotides (Usman et al., *J. Am. Chem. Soc.* 109:7845-7854 (1987)), and all the required 3'-O-phosphoramidites are commercially available. In addition, the use of aminomethylpolystyrene is preferred as the support material due to its advantageous properties (McCollum and Andrus *Tetrahedron Letters* 32:4069-4072 (1991)). Fluorescein can be added to the 5'-end of a substrate RNA during the synthesis by using commercially available fluorescein phosphoramidites. In general, a desired oligomer can be synthesized using a standard RNA cycle. Upon completion of the assembly, all base labile protecting groups are removed by an 8 hour treatment at 55°C with concentrated aqueous ammonia/ethanol (3:1 v/v) in a sealed vial. The ethanol suppresses premature removal of the 2'-O-TBDMS groups which would otherwise lead to appreciable strand cleavage at the resulting ribonucleotide positions under the basic conditions of the deprotection (Usman et al., *J. Am. Chem. Soc.* 109:7845-7854 (1987)). After lyophilization the TBDMS protected oligomer is treated with a mixture of triethylamine trihydrofluoride/triethylamine/N-methylpyrrolidinone for 2 hours at 60°C to afford fast and efficient removal of the silyl protecting groups under neutral conditions (Wincott et al., *Nucleic Acids Res.* 23:2677-

2684 (1995)). The fully deprotected oligomer can then be precipitated with butanol according to the procedure of Cathala and Brunel (*Nucleic Acids Res.* 18:201 (1990)). Purification can be performed either by denaturing polyacrylamide gel electrophoresis or by a combination of ion-exchange HPLC (Sproat *et al.*, *Nucleosides and Nucleotides* 14:255-273 (1995)) and reversed phase HPLC. For use in cells, it is preferred that synthesized oligomers be converted to their sodium salts by precipitation with sodium perchlorate in acetone. Traces of residual salts are then preferably removed using small disposable gel filtration columns that are commercially available. As a final step it is preferred that the authenticity of the isolated oligomers is checked by matrix assisted laser desorption mass spectrometry (Pieles *et al.*, *Nucleic Acids Res.* 21:3191-3196 (1993)) and by nucleoside base composition analysis. In addition, a functional cleavage test with the oligomer on the corresponding chemically synthesized short oligoribonucleotide substrate is also preferred.

Cleavage of RNA Substrates

The disclosed compositions have a very high *in vivo* activity since the RNA cleavage is promoted by protein factors that are present in the nucleus or cytoplasm of the cell. Examples of such protein factors which can increase the activity of hammerhead ribozymes are, for example, the nucleocapsid protein NCp7 of HIV1 (Müller *et al.*, *J. Mol. Biol.* 242:422-429 (1994)) and the heterogeneous nuclear ribonucleoprotein A1 (Heidenreich *et al.*, *Nucleic Acids Res.* 23:2223-2228 (1995)). Thus, long RNA transcripts can be cleaved efficiently within the cell by the disclosed compositions.

The disclosed compositions can be used in pharmaceutical compositions that contain one or several oligomers as the active substance, and, optionally, pharmaceutically acceptable auxiliary substances, additives and carriers. Such pharmaceutical compositions are suitable for the production of an agent to specifically inactivate the expression of genes in eukaryotes, prokaryotes and viruses, especially of human genes such as tumor genes or viral genes or RNA molecules in a cell. Further areas of application are the inactivation of the expression of plant genes or insect genes. Thus, the

disclosed compositions can be used as drugs for humans and animals as well as a pesticide for plants.

A variety of methods are available for delivering the disclosed compositions to cells. For example, in general, the disclosed compositions can be incorporated within or on microparticles. As used herein, microparticles include liposomes, virosomes, microspheres and microcapsules formed of synthetic and/or natural polymers. Methods for making microcapsules and microspheres are known to those skilled in the art and include solvent evaporation, solvent casting, spray drying and solvent extension. Examples of useful polymers which can be incorporated into various microparticles include polysaccharides, polyanhydrides, polyorthoesters, polyhydroxides and proteins and peptides.

Liposomes can be produced by standard methods such as those reported by Kim *et al.*, *Biochim. Biophys. Acta*, 728:339-348 (1983); Liu *et al.*, *Biochim. Biophys. Acta*, 1104:95-101 (1992); and Lee *et al.*, *Biochim. Biophys. Acta*, 1103:185-197 (1992); Wang *et al.*, *Biochem.*, 28:9508-9514 (1989)). Such methods have been used to deliver nucleic acid molecules to the nucleus and cytoplasm of cells of the MOLT-3 leukemia cell line (Thierry and Dritschilo, *Nucl. Acids Res.*, 20:5691-5698 (1992)). Alternatively, the disclosed compositions can be incorporated within microparticles, or bound to the outside of the microparticles, either ionically or covalently.

Cationic liposomes or microcapsules are microparticles that are particularly useful for delivering negatively charged compounds such as the disclosed compounds, which can bind ionically to the positively charged outer surface of these liposomes. Various cationic liposomes have previously been shown to be very effective at delivering nucleic acids or nucleic acid-protein complexes to cells both *in vitro* and *in vivo*, as reported by Felgner *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987); Felgner, *Advanced Drug Delivery Reviews*, 5:163-187 (1990); Clarenc *et al.*, *Anti-Cancer Drug Design*, 8:81-94 (1993). Cationic liposomes or microcapsules can be prepared using mixtures including one or more lipids containing a cationic side group in a sufficient quantity such that the liposomes or microcapsules

formed from the mixture possess a net positive charge which will ionically bind negatively charged compounds. Examples of positively charged lipids that may be used to produce cationic liposomes include the aminolipid dioleoyl phosphatidyl ethanolamine (PE), which possesses a positively charged primary amino head group; phosphatidylcholine (PC), which possess positively charged head groups that are not primary amines; and N[1-(2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium ("DOTMA," see Felgner *et al.*, *Proc. Natl. Acad. Sci USA*, 84:7413-7417 (1987); Felgner *et al.*, *Nature*, 337:387-388 (1989); Felgner, *Advanced Drug Delivery Reviews*, 5:163-187 (1990)).

A preferred form of microparticle for delivery of the disclosed compositions are heme-bearing microparticles. In these microparticles, heme is intercalated into or covalently conjugated to the outer surface of the microparticles. Heme-bearing microparticles offer an advantage in that since they are preferentially bound and taken up by cells that express the heme receptor, such as hepatocytes, the amount of drug required for an effective dose is significantly reduced. Such targeted delivery may also reduce systemic side effects that can arise from using relatively high drug concentrations in non-targeted delivery methods. Preferred lipids for forming heme-bearing microparticles are 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) and dioleoyl phosphatidyl ethanolamine (DOPE). The production and use of heme-bearing microparticles are described in PCT application WO 95/27480 by Innovir.

The disclosed compositions can also be encapsulated by or coated on cationic liposomes which can be injected intravenously into a mammal. This system has been used to introduce DNA into the cells of multiple tissues of adult mice, including endothelium and bone marrow, where hematopoietic cells reside (see, for example, Zhu *et al.*, *Science*, 261:209-211 (1993)).

Liposomes containing the disclosed compositions can be administered systemically, for example, by intravenous or intraperitoneal administration, in an amount effective for delivery of the disclosed compositions to targeted cells. Other possible routes include trans-dermal or oral, when used in

conjunction with appropriate microparticles. Generally, the total amount of the liposome-associated oligomer administered to an individual will be less than the amount of the unassociated oligomer that must be administered for the same desired or intended effect.

5 Compositions including various polymers such as the polylactic acid and polyglycolic acid copolymers, polyethylene, and polyorthoesters and the disclosed compositions can be delivered locally to the appropriate cells by using a catheter or syringe. Other means of delivering such compositions locally to cells include using infusion pumps (for example, from Alza
10 Corporation, Palo Alto, California) or incorporating the compositions into polymeric implants (see, for example, Johnson and Lloyd-Jones, eds., *Drug Delivery Systems* (Chichester, England: Ellis Horwood Ltd., 1987), which can effect a sustained release of the therapeutic compositions to the immediate area of the implant.

15 For therapeutic applications the active substance is preferably administered at a concentration of 0.01 to 10,000 $\mu\text{g/kg}$ body weight, more preferably of 0.1 to 1000 $\mu\text{g/kg}$ body weight. The administration can, for example, be carried out by injection, inhalation (for example as an aerosol), as a spray, orally (for example as tablets, capsules, coated tablets etc.),
20 topically or rectally (for example as suppositories).

 The disclosed compositions can be used in a method for the specific inactivation of the expression of genes in which an active concentration of the composition is taken up into a cell so that the composition specifically cleaves a predetermined RNA molecule which is present in the cell, the cleavage
25 preferably occurring catalytically. Similar compositions, which are described in U.S. Patent No. 5,334,711, have been used successfully in mice to inactivate a gene (Lyngstadaas *et al.*, *EMBO J.* 14:5224-5229 (1995)). This process can be carried out *in vitro* on cell cultures as well as *in vivo* on living organisms (prokaryotes or eukaryotes such as humans, animals or plants).

30 The disclosed compositions can also be used as RNA restriction enzymes to cleave RNA molecules (in, for example, cell free *in vitro* reactions). The disclosed compositions can also be used in a reagent kit for

the restriction cleavage of RNA molecules which contains, for example, an oligomer and suitable buffer substances. In this case the oligomer and the buffer substances can be present in the form of solutions, suspensions or solids such as powders or lyophilisates. The reagents can be present
5 together, separated from one another or optionally also on a suitable carrier. The disclosed compositions can also be used as a diagnostic agent or to identify the function of unknown genes.

The present invention will be further understood by reference to the following non-limiting examples.

10

Examples

Example 1: Cleavage reactions which indicate that an inosine substitution at position 15.1 can effectively cleave $N^{16.2}C^{16.1}H^{17}$.

A set of 12 substrates was synthesized which covered each
15 permutation of the $N^{16.2}C^{16.1}H^{17}$ motif where H^{17} is not guanosine. The oligomers and the corresponding substrates used in the cleavage assays are shown in Table 1. Each of the substrates was labeled with fluorescein at the 5' end and an inverted thymidine cap was used on the 3'-end. A set of four catalytic oligomers was synthesized, providing an appropriately matched
20 catalytic oligomer for each of the substrates. Each of these catalytic oligomers had an inosine at position 15.1. A control substrate and catalytic oligomer were also synthesized in which there was a U at position 16.1 of the substrate and an A at position 15.1 of the catalytic oligomer.

Table 1

$N^{16.2}N^{16.1}H^{17}$ Triplet		Substrate sequence	
5	ACC	FI-GAAUACCGGUCGC*T	(SEQ ID NO:4)
	ACA	FI-GAAUACAGGUCGC*T	(SEQ ID NO:5)
	ACU	FI-GAAUACUGGUCGC*T	(SEQ ID NO:6)
10	GCC	FI-GAAUGCCGGUCGC*T	(SEQ ID NO:7)
	GCA	FI-GAAUGCAGGUCGC*T	(SEQ ID NO:8)
	GCU	FI-GAAUGCUGGUCGC*T	(SEQ ID NO:9)
15	CCC	FI-GAAUCCCGGUCGC*T	(SEQ ID NO:10)
	CCA	FI-GAAUCCAGGUCGC*T	(SEQ ID NO:11)
	CCU	FI-GAAUCCUGGUCGC*T	(SEQ ID NO:12)
20	UCC	FI-GAAUCCCGGUCGC*T	(SEQ ID NO:13)
	UCA	FI-GAAUUCAGGUCGC*T	(SEQ ID NO:14)
	UCU	FI-GAAUUCUGGUCGC*T	(SEQ ID NO:15)
	GUC	FI-GAAUGUCGGUCGC*T	(SEQ ID NO:16)
Targeted triplet		Catalytic oligomer sequence	
25	ACH	gcgaccuGAuGaggccgugaggccGaaIuauc*T	(SEQ ID NO:17)
	GCH	gcgaccuGAuGaggccgugaggccGaaIcauc*T	(SEQ ID NO:18)
	CCH	gcgaccuGAuGaggccgugaggccGaaIgauc*T	(SEQ ID NO:19)
	UCH	gcgaccuGAuGaggccgugaggccGaaIaunc*T	(SEQ ID NO:20)
30	GUC	gcgaccuGAuGaggccgugaggccGaaAcauc*T	(SEQ ID NO:21)
FI = Fluorescein label *T = 3'-3' inverted thymidine A, C, G, I, U = ribonucleotides (I is inosine) a, c, g, u = 2'-O-allyl-ribonucleotides			

The above substrates and catalytic oligomers were used in cleavage reactions to determine the ability of an inosine at position 15.1 to overcome the requirement of a U at position 16.1 for cleavage. All of the reactions were performed using the following protocol. The reactions were typically done in 100 μ l and they contained distilled, autoclaved H_2O , 10 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4, 5 μ M ribozyme, and 0.25 μ M substrate. The catalytic oligomer, substrate, and buffer were added together and heated to

95°C for 5 minutes. After cooling to room temperature over 5 minutes the reactions were brought to 10 mM MgCl₂, mixed, and placed at 37°C. 10 μL aliquots were removed at specific time intervals (10, 30, 60, and 120 minutes) and added to 3 μl of loading buffer (95% formamide, 100 mM EDTA pH 8.0, 0.05% bromophenol blue) to quench the reaction. Samples were analyzed by 20% polyacrylamide gel electrophoresis. Gels were analyzed on a Molecular Dynamics Fluorescence Imager. The results of cleavage reactions of this type, using the substrates and catalytic oligomers shown in Table 1, are shown in Table 2.

		Table 2				
10	N ^{14,2} N ^{14,2} H ¹⁷ Triplet	After mixing	10	30	60	120
15	I ^{15,1} U ^{15,2} Catalytic oligomer					
	ACC	4.4	28.2	58.1	91.5	91.5
	ACA	7.7	71.8	84.7	93.1	94.8
	ACU	1.8			58.7	70.5
20	I ^{15,1} C ^{15,2} Catalytic oligomer					
	GCC	1.62	39.6	59.9	82.0	87.0
	GCA	13.7	65.3	78.7	89.7	93.1
25	GCU	—			64.3	74.8
	I ^{15,1} G ^{15,2} Catalytic oligomer					
	CCC	—			34.33	45.38
30	CCA	1.1	18.8	45.5	70.8	80.63
	CCU	2.0			28.4	36.7
	I ^{15,1} A ^{15,2} Catalytic oligomer					
35	UCC	6.8			57.0	64.7
	UCA	1.6			39.6	60.8
	UCU	3.3			41.1	53.1
	A ^{15,1} C ^{15,2} Catalytic oligomer					
40	GUC	1.6	38.5	66.5	93.5	

The numbers represent the percentage of substrate cleaved at the indicated time point (which were at 0, 10, 30, 60, and 120 minutes after starting the reaction). The results indicate that substrates with a C at position 16.1 are able to be cleaved by catalytic oligomers containing an I at position 15.1. While there are differences between the various substrates at the 120 minute time point, the data show that a substrate with a C at position 16.1 in conjunction with a catalytic oligomer with an I at position 15.1 is able to effectively cleave in all backgrounds, indicating that the substitution of an I at position 15.1 does in fact allow for the cleavage of any appropriate substrate containing a N^{14.2}C^{16.1}H¹⁷ site.

Initial rates of cleavage of the twelve substrates having C^{16.1}, and the control substrate having U^{16.1}, by the corresponding catalytic oligomers (all shown in Table 1) were determined using single turnover kinetics. Single turnover kinetics were assessed by mixing 2.5 μ l of a 100 μ M ribozyme solution, 2.5 μ l of a 10 μ M solution of 5' fluorescein labeled substrate, and 10 μ l of a 100 mM Tris-HCl pH 7.4 solution. The mixture was diluted to a final volume of 90 μ l, heated to 95°C for 5 minutes, and cooled to 37°C. The reaction was started by adding 10 μ l of a 100 mM MgCl₂ solution. The final concentrations of the reaction components were 250 nM substrate, 2.5 μ mol ribozyme, and 10 mM MgCl₂. Ten microliter samples were removed at various times and mixed with 10 μ l of a 100 mM EDTA, bromphenol blue solution to stop the reaction. Cleavage products were separated from unreacted substrate by PAGE and were quantitated on a Molecular Dynamics Fluorescence Imager.

The data, measured in fraction of substrate cleaved versus time, were fitted to the equation:

$$\text{frac}[P] = H_0(1 - e^{-k_2 t})/S_0$$

as described by Jankowsky and Schwenzler, *Nucl. Acids Res.* 24:433 (1996). The calculated values of k_2 for the various ribozymes are shown in Table 3.

Table 3

$N^{16,2}N^{16,1}H^{17}$			
Triplet			
gcgacccuGAuGaggccgugaggccGaaIuauc*T (SEQ ID NO:17)			
5		k_2 (min ⁻¹)	Substrate sequence
	ACC	0.07	FI-GAAUACCGGUCGC*T (SEQ ID NO:4)
	ACA	0.36	FI-GAAUACAGGUCGC*T (SEQ ID NO:5)
	ACU	0.026	FI-GAAUACUGGUCGC*T (SEQ ID NO:6)
10	gcgacccuGAuGaggccgugaggccGaaIcauc*T (SEQ ID NO:18)		
		k_2 (min ⁻¹)	Substrate sequence
	GCC	0.12	FI-GAAUGCCGGUCGC*T (SEQ ID NO:7)
15	GCA	0.48	FI-GAAUGCAGGUCGC*T (SEQ ID NO:8)
	GCU	0.05	FI-GAAUGCUGGUCGC*T (SEQ ID NO:9)
gcgacccuGAuGaggccgugaggccGaaIgauc*T (SEQ ID NO:19)			
20		k_2 (min ⁻¹)	Substrate sequence
	CCC	<0.01	FI-GAAUCCCGGUCGC*T (SEQ ID NO:10)
	CCA	0.04	FI-GAAUCCAGGUCGC*T (SEQ ID NO:11)
	CCU	<0.01	FI-GAAUCCUGGUCGC*T (SEQ ID NO:12)
25	gcgacccuGAuGaggccgugaggccGaaIaauuc*T (SEQ ID NO:20)		
		k_2 (min ⁻¹)	Substrate sequence
	UCC	<0.01	FI-GAAUCCCGGUCGC*T (SEQ ID NO:13)
	UCA	<0.01	FI-GAAUUCAGGUCGC*T (SEQ ID NO:14)
30	UCU	<0.01	FI-GAAUUCUGGUCGC*T (SEQ ID NO:15)
gcgacccuGAuGaggccgugaggccGaaAcauuc*T (SEQ ID NO:21)			
		k_2 (min ⁻¹)	Substrate sequence
35	GUC	0.13	FI-GAAUGUCGGUCGC*T (SEQ ID NO:16)
FI = Fluorescein label *T = 3'-3' inverted thymidine A, C, G, I, U = ribonucleotides (I is inosine) a, c, g, u = 2'-O-allyl-ribonucleotides			
40			

The results show that substrates with $A^{16,2}C^{16,1}H^{17}$ and $G^{16,2}C^{16,1}H^{17}$ triplets are cleaved at a high rate. Comparison to the control catalytic oligomer having an A at position 15.1 (to cleave a substrate with a

G^{16.2}U^{16.1}C¹⁷ triplet) shows that substrates with A^{16.2}C^{16.1}A¹⁷ and G^{16.2}C^{16.1}A¹⁷ triplets (to be cleaved by a catalytic oligomer with an I at position 15.1) have an initial rate of cleavage that is higher than the corresponding control reactions involving reactants with a standard A^{15.1}-U^{16.1} base pair.

5 **Example 2: Rate of cleavage at GCH and ACH triplets by catalytic oligomers containing only ribonucleotides**

Cleavage of substrate RNA by all-ribonucleotide versions of oligomers (no modifications) designed to cleave GCH and ACH triplets was assessed. The oligomers used were SEQ ID NO:17 (cleaves after ACH triplets) (Table 1) and SEQ ID NO:18 (cleaves after GCH triplets) (Table 1). The
10 corresponding short fluorescent labelled substrates, SEQ ID NOS:4, 5, and 6 (containing ACH triplets) (Table 1) and SEQ ID NOS:7, 8, and 9 (containing GCH triplets) (Table 1) were used with catalytic oligomers SEQ ID NO:17 and SEQ ID NO:18 respectively.

15 The reactions were performed under single-turnover kinetic conditions, using 2.5 μ M catalytic oligomer and 250 nM substrate, both at pH 6.0 in the presence of 10 mM Mg²⁺ and also at pH 7.4 in the presence of 1 mM Mg²⁺. All other reaction conditions were as in Example 1.

The data were analyzed as in Example 1. The k₂ values for the 6
20 combinations of all-ribonucleotide catalytic oligomer and corresponding substrates are shown in Table 4.

		Table 4	
		k ₂ (min) ⁻¹	
		pH 6.0; 10 mM Mg ²⁺	pH 7.4; 1 mM Mg ²⁺
25	Triplet		
	GCA	0.39	2.32
	GCC	0.18	2.03
	GCU	0.03	0.10
30	ACA	0.41	1.12
	ACC	0.17	0.71
	ACU	0.02	0.06

The all ribonucleotide oligomers targeting GCA, GCC, ACA, and ACC sites
35 have a higher rate of cleavage than the all ribonucleotide oligomers targeting GCU or ACU sites. Furthermore, Table 4 indicates the cleavage activity is

very high in the presence of 1 mM Mg^{2+} . This level of Mg^{2+} is similar to the concentration of Mg^{2+} *in vivo*.

Example 3: Rate of cleavage at GCH and ACH triplets by catalytic oligomers containing only 6 ribonucleotides

5 Cleavage of substrate RNA by 6-ribonucleotide versions of oligomers designed to cleave GCH and ACH triplets was assessed. In these oligomers, ribonucleotides were present at positions U⁴, G⁵, A⁶, G⁸, G¹² and I^{15,1} and all other positions were 2'-O-allyl-ribonucleotides (see Figure 2 for numbering). The oligomers used were SEQ ID NO:17 (cleaves after ACH triplets) (Table 1) and SEQ ID NO:18 (cleaves after GCH triplets) (Table 1). The
10 corresponding short fluorescent labelled substrates, SEQ ID NOS:4, 5, and 6 (containing ACH triplets) (Table 1) and SEQ ID NOS:7, 8, and 9 (containing GCH triplets) (Table 1) were used with catalytic oligomers SEQ ID NO:17 and SEQ ID NO:18 respectively.

15 The reactions were performed under single-turnover kinetic conditions, using 2.5 μ M catalytic oligomer and 250 nM substrate, both at pH 6.0 in the presence of 10 mM Mg^{2+} and also at pH 7.4 in the presence of 1 mM Mg^{2+} . All other reaction conditions were as in Example 1.

The data were analyzed as in Example 1. The calculated k_2 values for
20 the six combinations of 6-ribo catalytic oligomers and corresponding substrates are shown in Table 5.

		Table 5	
		k_2 (min) ⁻¹	
Triplet		pH 6.0; 10 mM Mg^{2+}	pH 7.4; 1 mM Mg^{2+}
25	GCA	0.11	0.87
	GCC	0.03	0.14
	GCU	0.03	0.11
30	ACA	0.18	1.06
	ACC	0.01	0.12
	ACU	0.02	0.08

These results indicate that catalytic oligomers such as SEQ ID NO:17
35 (cleaves after ACH triplets) (Table 1) and SEQ ID NO:18 (cleaves after GCH triplets) (Table 1) remain active when all of the ribonucleotides except

those at positions U⁴, G³, A⁶, G⁸, G¹² and I^{15.1} are modified. These results also indicate that catalytic oligomers are capable of cleaving substrates at physiological concentrations of Mg²⁺.

Example 4: Rate of cleavage at a GCA triplet by versions of a catalytic oligomer differing in the sugar modification at position U⁴

Cleavage of substrate RNA by oligomers with modifications at position U⁴ was assessed. These assays showed that oligomers that contain nuclease resistant modifications retain activity and that different modifications can be made at a given position of the catalytic oligomer while retaining activity. The catalytic oligomers were based on SEQ ID NO:18. Variant I was made up of SEQ ID NO:18 with all but five nucleotides modified with 2'-O-allyl-ribonucleotides (ribonucleotides were present at positions G³, A⁶, G⁸, G¹² and I^{15.1} and all other positions were 2'-O-allyl-ribonucleotides; see Figure 2 for numbering). Variant II was made up of SEQ ID NO:18, modified with 2'-O-allyl-ribonucleotides at all but six nucleotides (ribonucleotides were present at positions U⁴, G³, A⁶, G⁸, G¹² and I^{15.1} and all other positions were 2'-O-allyl-ribonucleotides). Variant III contained ribonucleotides at positions G³, A⁶, G⁸, G¹² and I^{15.1} and a 2'-amino-2'-deoxyuridine at position U⁴. All other bases were 2'-O-allyl-ribonucleotides.

The reactions were performed under single-turnover kinetic conditions, using 2.5 μ M catalytic oligomer and 250 nM substrate, both at pH 7.4 in the presence of 1 mM Mg²⁺. All other reaction conditions were as in Example 1.

The data were analyzed as in Example 1. The calculated k₂ values were 2.32 min⁻¹ for the all-ribonucleotide compound, 0.10 min⁻¹ for variant I, 0.87 min⁻¹ for variant II and 0.56 min⁻¹ for variant III respectively. These results indicate that different modifications which inhibit RNase A activity can be made at the highly RNase A sensitive U⁴ site while retaining activity. The nuclease resistant variant containing the 2'-amino-2'-deoxyuridine at position U⁴ is only marginally less active than the RNase A sensitive but highly active variant which contains a ribouridine at this position. This

demonstrates that it is possible to generate nuclease resistant catalytic oligomers with activities close to the unmodified all-ribonucleotide versions, and that different modifications can be made at a given site while retaining activity.

5 **Example 5: Comparison of cleavage activities of 2'-O-methyl- and 2'-O-allyl-modified catalytic oligomers at all 12 possible NCH triplets**

Cleavage of substrate RNA by oligomers with modifications in the nucleotides at all positions except G⁵, A⁶, G⁸, G¹², and I^{15,1} were assayed to
10 show cleavage at all NCH sites. Catalytic oligomers were based on SEQ ID NO:17 (cleaves after ACH triplets), SEQ ID NO:18 (cleaves after GCH triplets), SEQ ID NO:19 (cleaves after CCH triplets) and SEQ ID NO:20 (cleaves after UCH triplets) that either contained 2'-O-allyl-ribonucleotides or 2'-O-methyl-ribonucleotides at all positions except G⁵, A⁶, G⁸, G¹², and
15 I^{15,1} were synthesized. The respective fluorescent labelled substrates SEQ ID NOS:4, 5 and 6 (containing ACH triplets, cleaved by SEQ ID NO:17), SEQ ID NOS:7, 8 and 9 (containing GCH triplets, cleaved by SEQ ID NO:18), SEQ ID NOS:10, 11 and 12 (containing CCH triplets, cleaved by SEQ ID NO:19) and SEQ ID NOS: 13, 14 and 15 (containing UCH triplets, cleaved
20 by SEQ ID NO:20) were also synthesized. The sequences of SEQ ID NOS: 4-20 are shown in Table 1.

The reactions were performed under single-turnover kinetic conditions, using 2.5 μ M catalytic oligomer and 250 nM substrate, at pH 7.4 in the presence of 10 mM magnesium ions. All other reaction conditions
25 were as in Example 1. The data were analyzed as in Example 1.

The results from these assays indicated that catalytic oligomers directed at GCH, ACH, CCH, and UCH substrates are capable of cleaving their respective targets. The results also indicate that for many catalytic oligomer:substrate combinations there is virtually no difference between the
30 2'-O-allyl-ribonucleotide modified catalytic oligomers and the corresponding 2'-O-methyl-ribonucleotide catalytic oligomers. Furthermore, for no

combination is there much more than a 2-fold difference between these two types of modifications.

Example 6: Rate of cleavage at a GCA triplet by versions of a catalytic oligomer differing in the nucleobase at position N⁷

5 Cleavage assays were performed to show that different bases with different modifications could be substituted at position N⁷ and still retain activity. All catalytic oligomers contained 2'-O-allyl-ribonucleotides at all positions except G⁵, A⁶, G⁸, G¹² and I¹⁵,¹ which were ribonucleotides. All catalytic oligomers were designed to cleave after GCH triplets. The
10 sequences of the catalytic oligomers were as follows (the N⁷ position is marked in boldface):

	L-gcgacc <u>cu</u> GAnGaggccgugaggccGaalcaunc*T	(SEQ ID NO:18)
	L-gcgacc <u>cu</u> GAcGaggccgugaggccGaalcaunc*T	(SEQ ID NO:22)
15	L-gcgacc <u>cu</u> GAnGaggccgugaggccGaalcaunc*T	(SEQ ID NO:23)
	L-gcgacc <u>cu</u> GAgGaggccgugaggccGaalcaunc*T	(SEQ ID NO:24)
	L-gcgacc <u>cu</u> GAlGaggccgugaggccGaalcaunc*T	(SEQ ID NO:25)
	L-gcgacc <u>cu</u> GaqGaggccgugaggccGaalcaunc*T	(SEQ ID NO:26)
	L-gcgacc <u>cu</u> GAnGaggccgugaggccGaalcaunc*T	(SEQ ID NO:27)

20

L = 5'-terminal hexanediol linker

*T = 3'-3' inverted thymidine

A, G and I are ribonucleotides

a, c, g, u and i are 2'-allyloxy-2'-deoxyribonucleotides

25 q is 1-(2-O-allyl-β-D-ribofuranosyl)quinazoline-2,4-dione

n is 5-nitro-1-(2-O-allyl-β-D-ribofuranosyl)indole

The reactions were performed under single-turnover kinetic conditions, using 2.5 μM catalytic oligomer and 250 nM substrate, at pH 7.4
30 in the presence of 10 mM magnesium ions. All other reaction conditions were as in Example 1. The data were analyzed as in Example 1.

Graphs showing fraction product versus time curves of the N⁷ variant oligomers are shown in Figures 4A and 4B. The data indicate that all variants at N⁷ are capable of cleaving very well. The catalytic oligomers
35 tolerate bulky groups such as the uracil analogue, quinazoline-2,4-dione quite well. Information such as this is important because it shows that there is a position in the catalytic oligomers which can be varied to optimize catalytic structure for a given substrate.

Example 7: *In vitro* cleavage of a long substrate derived from hepatitis C virus

A long RNA substrate transcribed from a plasmid was used to demonstrate cleavage of such a substrate using the disclosed compositions.

- 5 Plasmid pN(1-4728) contains the first 1358 bases of the hepatitis C virus genome (HCV). The sequence is oriented so as to allow a runoff transcript of the *Bam* HI-linearized plasmid that produces a 1358 base transcript. A runoff transcription (3-5 μ g) was performed in a 100 μ l reaction volume containing 40 mM Tris-HCl (pH 7.5), 18 mM $MgCl_2$, 1 mM spermidine, 5
- 10 mM DTT, 2000 U/ml placental RNase inhibitor (Promega), 3 mM of each of ATP, UTP, CTP and GTP, 50 μ Ci of [α - 32 P]GTP (DuPont NEN) and 3000 U/ml T7 RNA polymerase (New England Biolabs). The reaction was incubated at 37°C for 2-3 hours and then terminated by addition of 100 μ l RNA gel-loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene
- 15 cyanol FF and 0.025% bromophenol blue). The mixture was then heated at 90°C for three minutes, snap-cooled on ice and subjected to electrophoresis on a 4% polyacrylamide/7 M urea gel. The 1358 nucleotide long transcript was visualized by UV shadowing, the band was excised and the RNA extracted by electroelution for one hour. The RNA was then recovered by
- 20 overnight ethanol precipitation. After centrifugation and washing with 70% ethanol the purified transcript was resuspended in 20 μ l of DEPC sterilized water and the concentration was determined by UV measurement.

The following oligomers were designed to target the runoff transcript of the HCV.

- 25 L-gganucgtcGAuGaggccgugaggccGaalcucaugg*T (SEQ ID NO:28)
 L-gauncgtcGAaGaggccgugaggccGaalcucaugg*T (SEQ ID NO:29)
 L-ggamutgcUGAuGaggccgugaggccGaalcucaugg*T (SEQ ID NO:30)
 L-gauncgtcGAuGaggccgugaggccGaalcucaugg*T (SEQ ID NO:31)

- 30 L = 5'-terminal hexanediol linker
 *T = 3'-3' inverted thymidine
 A, G, U and I are ribonucleotides
 a, c, g and u are 2'-allyloxy-2'-deoxyribonucleotides
- 35

These sequences are targeted to the GCA-345 site of the HCV genome which is present in the transcript.

Cleavage reactions were carried out in a 10 μ l volume containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 30 nM radiolabeled transcript and 300 nM catalytic oligomer (either SEQ ID NOS:28-31). Incubation was performed at 37°C for 10 minutes and 60 minutes for each oligomer tested, and the reactions were quenched by addition of 10 μ l of gel-loading buffer containing 20 mM EDTA, heated at 90°C for 5 minutes and then cooled on ice. Uncleaved transcript and cleavage products were then separated by electrophoresis on a 4% polyacrylamide/7 M urea gel. After electrophoresis the gel was transferred onto Whatman 3MM filter paper and dried for two hours at 80°C. Bands were quantitated by exposure to a PhosphorImager screen.

The results of these assays indicated that all four of the catalytic oligomers were capable of cleaving the HCV runoff transcript. This indicates that target catalytic oligomers containing all 2'-O-allyl-ribonucleotides, except at positions U⁴, G⁵, A⁶, G⁸, G¹² and I^{15.1} (SEQ ID NO:30) and except at positions G⁵, A⁶, G⁸, G¹² and I^{15.1} (SEQ ID NOS:28, 29, and 31), are capable of cleaving a long RNA.

Example 8: Comparison of *in vitro* cleavage of a long substrate derived from hepatitis C virus using catalytic oligomers targeting GCA and GUA triplets

Cleavage assays were performed to show that catalytic oligomers designed to cleave the 1358 base HCV substrate function at concentrations as low as 30 nM. The preparation of the runoff transcript was as in Example 7. The oligomers used in these assays were as follows:

L-ggauncgcuGAuGaggccgugaggccGaaIcucaugg*T (SEQ ID NO:28)
L-uuggugucuGAuGaggccgugaggccGaaAcgunugg*T (SEQ ID NO:32)

L = 5'-terminal hexanediol linker

*T = 3'-3' inverted thymidine

A, G, U and I are ribonucleotides

a, c, g and u are 2'-allyloxy-2'-deoxyribonucleotides

These catalytic oligomers target the GCA 345 triplet (oligomer of SEQ ID NO:28) and the GUA 378 triplet (oligomer of SEQ ID NO:32). The fragments obtained when analyzing cleavage reactions of the 1358 runoff transcript of the HCV are 347 and 1011 nucleotides long for cleavage at the GCA 345 site and 380 and 978 nucleotides long for cleavage at the GUA 378 site. The catalytic oligomers were compared at 1 and 3 μ M with a reaction time of one hour and at concentrations of 30 nM, 100 nM, 300 nM, 1 μ M and 3 μ M with a reaction time of three hours. All other reaction and conditions were as in Example 7.

An analysis of the data indicated that the oligomers cleaved the HCV transcript after three hours at all concentrations of catalytic oligomer tested. This indicates that concentrations of catalytic oligomer of 30 nM are capable of cleaving a 1358 base RNA fragment of the HCV genome.

Example 9: Cleavage of human IL-2 mRNA in Jurkat cell lysates

Cleavage assays were performed to show that catalytic oligomers targeted to IL-2 mRNA were capable of cleaving the native mRNA in a cell lysate solution. These assays were performed with the catalytic oligomer of sequence:

L-gacuuagcuGAuGaggccgugaggccGaalcaugca*T (SEQ ID NO:33)

L = 5'-terminal hexanediol linker

*T = 3'-3' inverted thymidine

A, G, U and I are ribonucleotides

a, c, g and u are 2'-allyloxy-2'-deoxyribonucleotides.

This sequence was designed to cleave after the GCA, where the A is at position 140, in the human interleukin-2 mRNA (sequence name HSIL2R in the EMBL Nucleotide Sequence Database 43rd Edition).

Jurkat cells were stimulated for five hours with phorbol 12-myristate 13-acetate (PMA)/phytohemagglutinin (PHA) to induce expression of IL-2. Crude cell lysates were then prepared by freeze-thawing as follows: 2×10^7 cells were washed in phosphate buffered saline (PBS) and resuspended in 500 μ l of RNase free deionized water, incubated at room temperature for 10 minutes, then snap frozen in liquid nitrogen and thawed at 37°C. Cell debris

was removed by low speed centrifugation leaving a crude lysate for use in cleavage assays.

Each cleavage reaction was carried out at 37°C in a 100 µl reaction volume including 62.5 µl of cell lysate, 50 mM Tris pH 7.5, 10 mM MgCl₂ and 1 µM catalytic oligomer for the reaction times: 0, 10, 30, 60, and 120 minutes. The following controls were done, control after 120 minutes with no oligomer, IL-2 probe control, IL-2 and β-actin RNase digested, and a control after 10 minutes with no oligomer. RNA was purified from the reactions using BioGene X-Cell solution and analyzed by a ribonuclease protection assay (RPA), using the RPA II kit from Ambion, following the manufacturer's protocol. Biotin labelled antisense RNA probes for IL-2 and β-actin RNA (internal standard) were prepared using an SP6 transcription kit. The template for the β-actin probe was purchased from Ambion and produced an RNA probe of 334 nucleotides and a protected fragment of 245 nucleotides in length.

An IL-2 probe template was made by using RT-PCR to amplify a fragment of the IL-2 sequence from Jurkat cell RNA. One primer was designed to also include the SP6 transcription promoter site so that the resultant DNA probe could be transcribed directly from the PCR reaction. The probe was designed to be 487 nucleotides in length, leading to a protected fragment also of 487 nucleotides after RPA analysis of IL-2 RNA. RPA analysis after cleavage with the catalytic oligomer should identify protected fragments of 428 and 59 nucleotides in addition to the full length RNA.

Protected RNA fragments were separated by polyacrylamide gel electrophoresis (5%), blotted onto nylon membrane and visualized by chemiluminescent detection using the BrightStar Biodetect kit from Ambion. Biotinylated RNA markers of lengths 500, 400, 300 and 200 nucleotides were used. The cell lysate prepared as described above contains, the IL-2 mRNA produced through intracellular transcription. This cell lysate, contains the targeted substrate. The product of the reaction between the substrate and the catalytic oligomer produces a 428 base and 59 base fragment of the IL-2

mRNA. By using a labeled probe for this sequence, the cleavage products can be detected. The IL-2 mRNA was cleaved by the catalytic oligomer, SEQ ID NO:33, in the presence of the cell lysate. This indicates that the catalytic oligomers are capable of cleaving a long, native, mRNA in the presence of the cellular material associated with the mRNA *in vivo*.

Example 10: Cleavage of rat dopamine D2 receptor RNA in CHO cell lysates

Cleavage assays were performed to show that catalytic oligomers targeted to rat dopamine D2 receptor mRNA were capable of cleaving the native mRNA in a CHO cell lysate solution. The assays were performed with the catalytic oligomer of sequence:

L- gcucgaccuGAuGaggccgugaggccGaalcugcgcu*T (SEQ ID NO:34)

L = 5'-terminal hexanediol linker
 *T = 3'-3' inverted thymidine
 A, G, U and I are ribonucleotides
 a, c, g and u are 2'-allyloxy-2'-deoxyribonucleotides.

This sequence was designed to cleave after the GCA, where the A is at position 811, in the rat dopamine D2 receptor RNA (sequence name RND2DOPR in the EMBL Nucleotide Sequence Database 43rd Edition).

Crude cell lysates were made from CHO cells stably transfected with the rat dopamine D2 receptor gene by the freeze-thawing procedure as described in Example 9 above. Each cleavage reaction was carried out at 37°C in a 100 µl reaction volume including 62.5 µl of cell lysate, 50 mM Tris pH 7.5, 10 mM MgCl₂, and 0.5 µM catalytic oligomer for the reaction times: 10, 30, 60, and 120 minutes. The following controls were performed: dopamine D2 receptor RNA and β-actin digested with RNase, dopamine D2 receptor RNA and β-actin control, rat dopamine D2 receptor RNA probe and β-actin control, control after 10 minutes with no oligomer, control after 30 minutes with no oligomer, and control after 60 minutes with no oligomer.

RNA was purified from the reactions and analyzed by ribonuclease protection assay, using the RPA II kit from Ambion and following the manufacturer's protocol. Biotin labelled antisense RNA probes for rat

dopamine D2 receptor and mouse β -actin RNA (internal standard) were prepared using an SP6 transcription kit. The template for the β -actin probe was purchased from Ambion and produced an RNA probe of 334 nucleotides and a protected fragment of 245 nucleotides in length.

- 5 A dopamine D2 receptor probe template was made by using RT-PCR to amplify a fragment of the dopamine D2 receptor sequence from CHO cell RNA. One primer was designed to also include the SP6 transcription promoter site so that the resultant DNA probe could be transcribed directly from the PCR reaction. The probe was designed to be 663 nucleotides after
- 10 RPA analysis of dopamine D2 receptor RNA. RPA analysis after cleavage with the catalytic oligomer should identify protected fragments of 394 and 269 nucleotides in addition to the full length RNA. Protected RNA fragments were separated by polyacrylamide gel electrophoresis (5%), blotted onto nylon membrane and visualized by chemiluminescent detection using the
- 15 BrightStar Biodetect kit from Ambion.

The results indicated that the D2 receptor RNA is cleaved by the catalytic oligomer, SEQ ID NO:34. The cleavage products were detectable by 10 minutes and increased with time indicating that the catalytic oligomer is not being substantially degraded over time.

20 **Example 11: Cleavage of human ICAM-1 mRNA in A549 cell lysates**

Cleavage assays were performed to show that catalytic oligomers targeted to ICAM-1 mRNA were capable of cleaving the native mRNA in an A549 cell lysate solution. The assays were performed with the catalytic oligomers of sequences:

- 25 L-ugguucucuGAuGaggccgugaggccGaaluguaanaa*T (SEQ ID NO:35)
L-uguaguccuGAuGaggccgugaggccGaaluanuucu*T (SEQ ID NO:36)

- 30 L = 5'-terminal hexanediol linker
*T = 3'-3' inverted thymidine
A, G, U and I are ribonucleotides
a, c, g and u are 2'-allyloxy-2'-deoxyribonucleotides.

- 35 These sequences were designed to cleave after the ACA sites where the first A is positioned at base 1205 (SEQ ID NO:35) and position 1592

(SEQ ID NO:36), in the human intercellular adhesion molecule-1 mRNA (ICAM-1) (sequence name HSICAM01 in the EMBL Nucleotide Sequence Database 43rd Edition).

Crude cell lysates were made from A549 cells, after a five hour
5 stimulation with 10 ng/ml of hTNF α to induce expression of ICAM-1, by the
freeze-thawing procedure as described in Example 9 above. Each cleavage
reaction was carried out for two hours at 37°C in a 100 μ l reaction volume
including 62.5 μ l of cell lysate, 50 mM Tris pH 7.5, 70 mM MgCl₂ and
catalytic oligomer to a final concentration of either 500 nM oligomer, 200
10 nM oligomer, 100 nM oligomer, 50 nM oligomer, control without catalytic
oligomer. RNA was purified from the reactions and analyzed by
ribonuclease protection assay, using the RPA II kit from Ambion, following
the manufacturer's protocol. Biotin labelled antisense RNA probes for
human ICAM-1 and GAPDH RNA (internal standard) were prepared using
15 an SP6 transcription kit. The template for the GAPDH probe was purchased
from Ambion and produced a protected fragment of 316 nucleotides in
length.

An ICAM-1 probe template was made by using RT-PCR to amplify a
fragment of the ICAM-1 sequence from A549 cell RNA. One primer was
20 designed to also include the SP6 transcription promoter site so that the
resultant DNA probe could be transcribed directly from the PCR reaction.
The probe was designed to be 598 nucleotides in length, leading to a
protected fragment also of 598 nucleotides after RPA analysis of ICAM-1
RNA. RPA analysis after cleavage with the catalytic oligomers should
25 identify protected fragments of 552 and 46 nucleotides (1205 ACA site) and
433 and 165 nucleotides (1592 ACA site) respectively in addition to the full
length RNA.

Protected RNA fragments were separated by 5% polyacrylamide gel
electrophoresis, blotted onto nylon membrane and visualized by
30 chemiluminescent detection using the BrightStar Biodetect kit from Ambion.
Biotinylated RNA markers of length 500, 400, 300 and 200 nucleotides were
used. Cleavage products were produced at all concentrations of oligomer

tested. Cleavage at the 1592 ACA site was particularly effective even using only 50 nM catalytic oligomer.

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: Innovir Laboratories, Inc.
 - (ii) TITLE OF INVENTION: Compositions Having RNA-Cleavage Activity
 - (iii) NUMBER OF SEQUENCES: 36
 - (iv) CORRESPONDENCE ADDRESS:
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1201 West Peachtree Street
 - (C) CITY: Atlanta
 - (D) STATE: GA
 - (E) COUNTRY: USA
 - (F) ZIP: 30309-3450
 - (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
 - (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
 - (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Pabst, Patrea L.
 - (B) REGISTRATION NUMBER: 31,284
 - (C) REFERENCE/DOCKET NUMBER: ILI 123
 - (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (404)-873-8794
 - (B) TELEFAX: (404)-873-8795

- (2) INFORMATION FOR SEQ ID NO: 1:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 57 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: circular
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

NNNNNNNNNN NNNNNNCUGA NGANNNNNNN NNNNNNNNYNG AARNNNNNNN NNNNNNUH

57

- (2) INFORMATION FOR SEQ ID NO: 2:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

NNNNNNCUGA NGANNNNNNN NNNNNNYNGAA NNNNN

35

- (2) INFORMATION FOR SEQ ID NO: 3:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

NNNNCHNNNN NN

12

- (2) INFORMATION FOR SEQ ID NO: 4:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
GAAUACCGGU CGCT 14
- (2) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
GAAUACAGGU CGCT 14
- (2) INFORMATION FOR SEQ ID NO: 6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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- (2) INFORMATION FOR SEQ ID NO: 7:
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(C) STRANDEDNESS: single
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(B) TYPE: nucleic acid
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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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(A) LENGTH: 14 base pairs

- (B) TYPE: nucleic acid
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(B) TYPE: nucleic acid
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- (2) INFORMATION FOR SEQ ID NO: 16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

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35

(2) INFORMATION FOR SEQ ID NO: 19:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

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35

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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GCGACCCUGA UGAGGCCGUG AGGCCGAANA AUUCT

35

(2) INFORMATION FOR SEQ ID NO: 21:

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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35

(2) INFORMATION FOR SEQ ID NO: 22:

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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35

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23

GCGACCCUGA AGAGGCCGUG AGGCCGAANC AUUCT

35

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24
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(B) TYPE: nucleic acid
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- (2) INFORMATION FOR SEQ ID NO: 26:
(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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GCGACCCUGA NGAGGCCGUG AGGCCGAANC AUUCT 35
- (2) INFORMATION FOR SEQ ID NO: 27:
(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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- (2) INFORMATION FOR SEQ ID NO: 28:
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(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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- (2) INFORMATION FOR SEQ ID NO: 31:
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- (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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UUGGUGUCUG AUGAGGCCGU GAGGCCGAAN CGUUGGT 38
- (2) INFORMATION FOR SEQ ID NO: 33:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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GACUAGCUG AUGAGGCCGU GAGGCCGAAN CAADGCAT 38
- (2) INFORMATION FOR SEQ ID NO: 34:
(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
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GCUCGACCUG AUGAGGCCGU GAGGCCGAAN CUGCGCUT 38
- (2) INFORMATION FOR SEQ ID NO: 35:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35
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- (2) INFORMATION FOR SEQ ID NO: 36:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36
UGUAGUCCUG AUGAGGCCGU GAGGCCGAAN UAUUCUT 38

Claims

1. A composition that cleaves an RNA substrate, the composition comprising components (a) and (b),

wherein component (a) comprises 5'-Z₁-Z₂-3' and component (b) comprises 5'-Z₃-Z₄-3', wherein components (a) and (b) either are separate molecules or are covalently coupled,

wherein Z₁ and Z₄ are oligomeric sequences which (1) are comprised of nucleotides, nucleotide analogues, or both, or (2) are oligonucleotide analogues, wherein the oligomeric sequences specifically interact with the RNA substrate by hybridization,

wherein Z₂ consists of

5'-X³X⁴X⁵X⁶X⁷X⁸-3', or

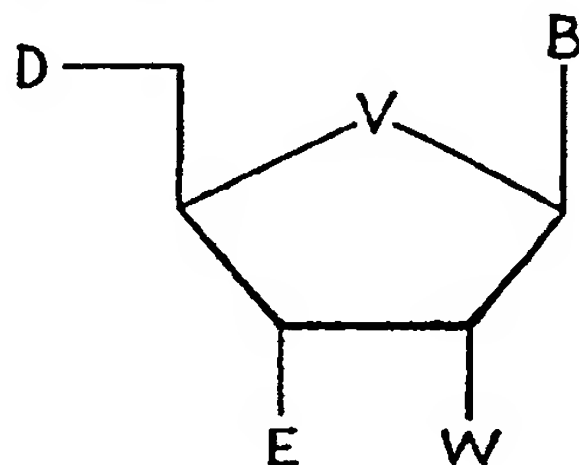
5'-X³X⁴X⁵X⁶X⁷X⁸X⁹-3',

wherein Z₃ consists of

5'-X¹²X¹³X¹⁴X^{15.1}-3', or

5'-X¹²X¹³X¹⁴X^{15.1}-3',

wherein Z₂ and Z₃ are comprised of nucleotides, nucleotide analogues, or both, wherein the nucleotides and nucleotide analogues each have the structure



wherein each B is independently adenin-9-yl, cytosin-1-yl, guanin-9-yl, uracil-1-yl, uracil-5-yl, hypoxanthin-9-yl, thymine-1-yl, 5-methylcytosin-1-yl, 2,6-diaminopurin-9-yl, purin-9-yl, 7-deazaadenin-9-yl, 7-deazaguanin-9-yl, 5-propynylcytosin-1-yl, 5-propynyluracil-1-yl, isoguanin-9-yl, 2-aminopurin-9-yl, 6-methyluracil-1-yl, 4-thiouracil-1-yl, 2-pyrimidone-1-yl, quinazoline-2,4-dione-1-yl, xanthin-9-yl, N²-dimethylguanin-9-yl, or a functional equivalent thereof,

wherein each V is independently an O, S, NH, or CH₂ group,

wherein each W is independently selected from the group consisting of H, -OH, -COOH, -CONH₂, -CONHR¹, -CONR¹R², -NH₂, -NHR¹, -NR¹R², -NHCOR¹, -SH, SR¹, -F, -ONH₂, -ONHR¹, -ONR¹R², -NHOH, -NHOR¹, -NR²OH, -NR²OR¹, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkyl, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyl, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkynyl, substituted or unsubstituted C₁-C₁₀ straight chain or branched alkoxy, substituted or unsubstituted C₂-C₁₀ straight chain or branched alkenyloxy, and substituted or unsubstituted C₂-C₁₀ straight chain or branched alkynyloxy, wherein the substituents are independently halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto, wherein R¹ and R² are, independently, substituted or unsubstituted alkyl, alkenyl, or alkynyl groups, where the substituents are independently halogen, cyano, amino, carboxy, ester, ether, carboxamide, hydroxy, or mercapto,

wherein D and E are residues which together form a phosphodiester or phosphorothioate diester bond between adjacent nucleosides or nucleoside analogues or together form an analogue of an internucleosidic bond,

wherein in X^{15,1}, B is hypoxanthin-9-yl or a functional equivalent thereof,

wherein in X³, X⁶, and X¹², B is independently guanine-9-yl, hypoxanthin-9-yl or 7-deazaguanine-9-yl;

wherein in X⁶, X⁹, X¹³, and X¹⁴, B is independently adenine-9-yl, 2,6-diaminopurine-9-yl, purine-9-yl or 7-deazaadenine-9-yl;

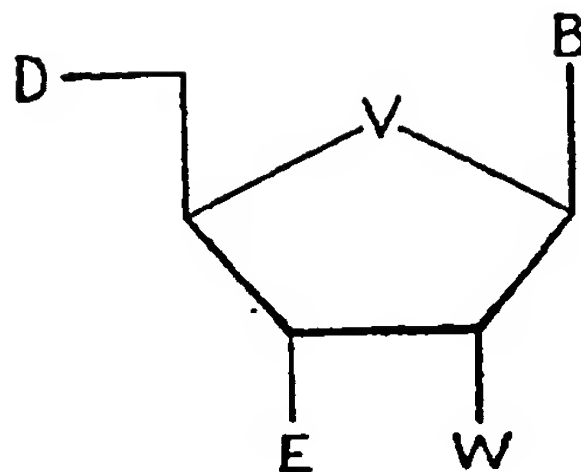
wherein in X⁴, B is uracil-1-yl, uracil-5-yl, thymine-1-yl or 5-propynyluracil-1-yl;

wherein in X³, B is cytosine-1-yl, 5-methylcytosine-1-yl or 5-propynylcytosine-1-yl;

wherein in X⁷, X^{9a}, and X¹², B is independently adenine-9-yl, cytosine-1-yl, guanine-9-yl, uracil-1-yl, uracil-5-yl, hypoxanthine-9-yl, thymine-1-yl, 5-methylcytosine-1-yl, 2,6-diaminopurine-9-yl, purine-9-yl, 7-deazaadenine-9-yl, 7-deazaguanine-9-yl, 5-propynylcytosine-1-yl, 5-propynyluracil-1-yl, isoguanine-9-yl, 2-aminopurine-9-yl, 6-methyluracil-1-yl, 4-thiouracil-1-yl, 2-pyrimidone-1-yl,

quinazoline-2,4-dione-1-yl, xanthin-9-yl, N²-dimethylguanin-9-yl, or a functional equivalent thereof.

2. The composition of claim 1 wherein the RNA substrate comprises 5'-Z₄'-C^{14,1}-X¹⁷-Z₁'-3',
wherein Z₁' and Z₄' interact with Z₁ and Z₄, wherein C^{14,1} is cytidine,
wherein X¹⁷ is adenosine, guanosine, cytidine, or uridine,
wherein cleavage occurs at the 3' phosphate of X¹⁷.
3. The composition of claim 2 wherein X¹⁷ is adenosine, cytidine, or uridine.
4. The composition of claim 1 wherein components (a) and (b) are covalently coupled to form a structure 5'-Z₁-Z₂-Z₃-Z₄-3',
wherein Z₃ is a linker.
5. The composition of claim 4 wherein the linker is selected from the group consisting of oligomeric sequences, non-nucleotide linkers, and a combination of oligomeric sequences and non-nucleotide linkers.
6. The composition of claim 5 wherein the oligomeric sequences independently either (a) contain nucleotides or nucleotide analogues or (b) are oligonucleotide analogues.
7. The composition of claim 1 wherein components (a) and (b) are separate molecules,
wherein component (a) comprises 5'-Z₁-Z₂-Z₄-3',
wherein component (b) comprises 5'-Z₁-Z₃-Z₄-3',
wherein Z₄ and Z₁ are oligomeric sequences which (1) are comprised of nucleotides, nucleotide analogues, or both, or (2) are oligonucleotide analogues,
wherein the oligomeric sequences specifically interact with each other.
8. The composition of claim 1, wherein Z₁ and Z₄ do not contain any pyrimidines that are ribonucleotides.
9. The composition of claim 1, wherein Z₁ and Z₄ do not contain any ribonucleotides.
10. The composition of claim 1, wherein Z₁ and Z₄ are comprised of nucleotides, nucleotide analogues, or both, wherein the nucleotides and nucleotide analogues each have the structure



wherein each B is independently adenin-9-yl, cytosin-1-yl, guanin-9-yl, uracil-1-yl, uracil-5-yl, hypoxanthin-9-yl, thymine-1-yl, 5-methylcytosin-1-yl, 2,6-diaminopurin-9-yl, purin-9-yl, 7-deazaadenin-9-yl, 7-deazaguanin-9-yl, 5-propynylcytosin-1-yl, 5-propynyluracil-1-yl, isoguanin-9-yl, 2-aminopurin-9-yl, 6-methyluracil-1-yl, 4-thiouracil-1-yl, 2-pyrimidone-1-yl, quinazoline-2,4-dione-1-yl, xanthin-9-yl, N²-dimethylguanin-9-yl, or a functional equivalent thereof,

wherein each V is independently an O, S, NH, or CH₂ group,

wherein each W is independently selected from the group consisting of substituted or unsubstituted C₁-C₁₀ straight chain or branched alkyl, C₂-C₁₀ straight chain or branched alkenyl, C₂-C₁₀ straight chain or branched alkynyl, C₁-C₁₀ straight chain or branched alkoxy, C₂-C₁₀ straight chain or branched alkenyloxy, and C₂-C₁₀ straight chain or branched alkynyloxy,

wherein D and E are residues which together form a phosphodiester or phosphorothioate diester bond between adjacent nucleosides or nucleoside analogues or together form an analogue of an internucleosidic bond.

11. The composition of claim 1, wherein Z₁ and Z₄ each independently contain from 3 to 40 nucleotides, nucleotide analogues, or a combination.

12. The composition of claim 1, wherein Z₂, Z₃, or both contain one or several nucleotide analogues wherein each W is independently selected from the group consisting of C₁-C₃ straight chain or branched alkyl, C₂-C₃ straight chain or branched alkenyl, C₂-C₃ straight chain or branched alkynyl, C₁-C₃ straight chain or branched alkoxy, C₂-C₃ straight chain or branched alkenyloxy, and C₂-C₃ straight chain or branched C₂-C₃ alkynyloxy.

13. The composition of claim 1, wherein each free 3' end is protected against exonuclease degradation.

14. The composition of claim 1, wherein in each X^3 , X^4 , X^7 and X^{12} , W is independently NH_2 , OH-substituted C_1-C_4 alkyl, OH-substituted C_2-C_4 alkenyl, OH-substituted C_1-C_4 alkoxy or OH-substituted C_2-C_4 alkenyloxy.
15. The composition of claim 14, wherein in each X^3 , X^4 , X^7 and X^{12} , W is independently NH_2 , methoxy, 2-hydroxyethoxy, allyloxy or allyl.
16. The composition of claim 1, wherein X^{12} is a ribonucleotide.
17. The composition of claim 1, wherein X^{13} and X^{14} , or a combination is a nucleotide analogue in which each W is independently C_1-C_4 alkyl, C_2-C_4 alkenyl, C_1-C_4 alkoxy, C_2-C_4 alkenyloxy, OH-substituted C_1-C_4 alkyl, OH-substituted C_2-C_4 alkenyl, OH-substituted C_1-C_4 alkoxy, or OH-substituted C_2-C_4 alkenyloxy.
18. The composition of claim 17, wherein X^{13} and X^{14} , or a combination is a nucleotide analogue in which each W is independently methoxy, 2-hydroxyethoxy or allyloxy.
19. The composition of claim 1, wherein $X^{15,1}$ is a ribonucleotide.
20. A method for the specific cleavage of an RNA substrate, the method comprising bringing into contact the composition of claim 1 and the RNA substrate.
21. A method of identifying the function of a gene, the method comprising
bringing into contact the composition of claim 1 and a cell containing the gene, wherein the composition reduces expression of the gene, and
observing any change in the cell.
22. A method of treating a disease that is associated with an RNA molecule, the method comprising administering to a subject having the disease the composition of claim 1, wherein the RNA substrate is the RNA molecule associated with the disease.
23. The method of claim 22 wherein the RNA molecule is an RNA molecule that is overexpressed.

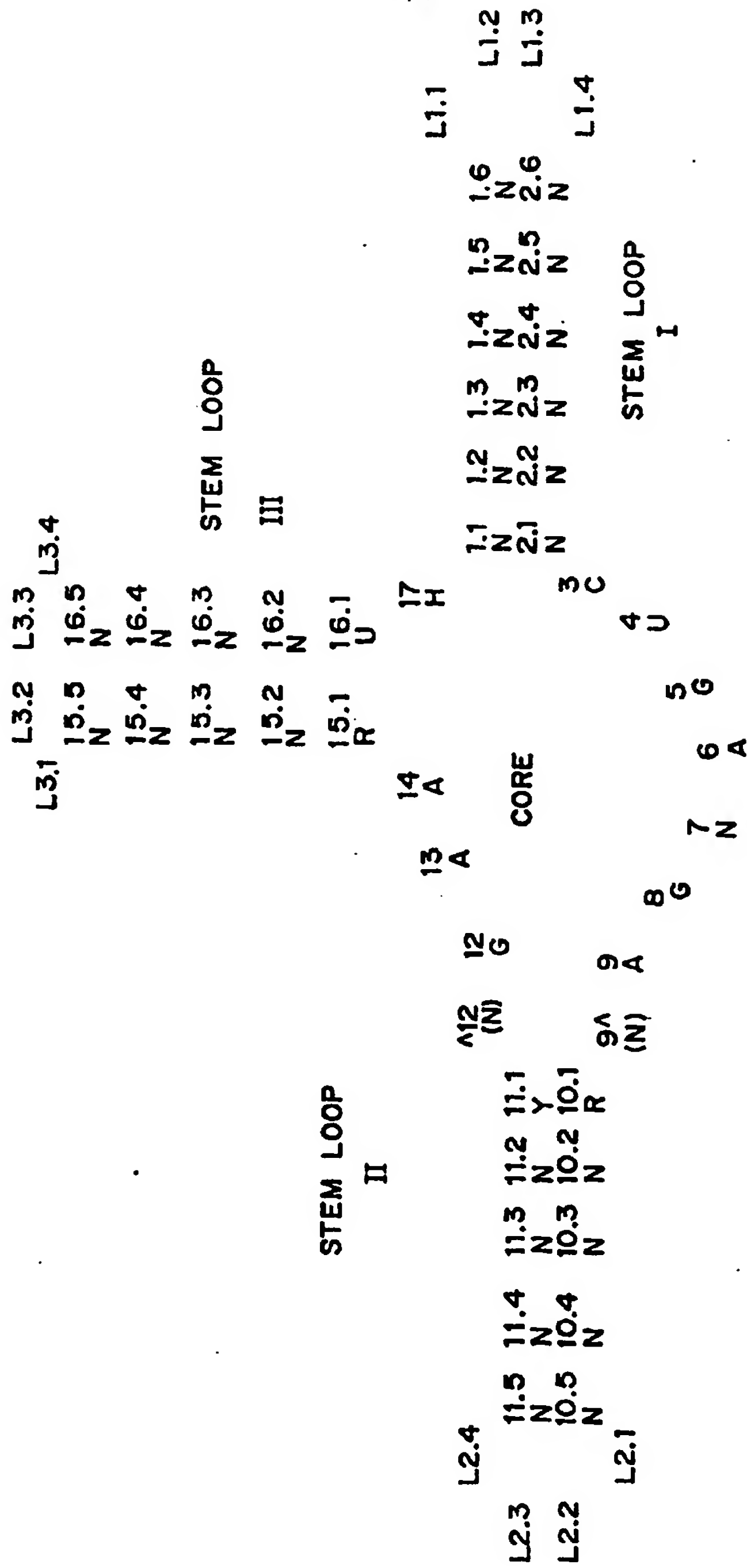


FIG. 1

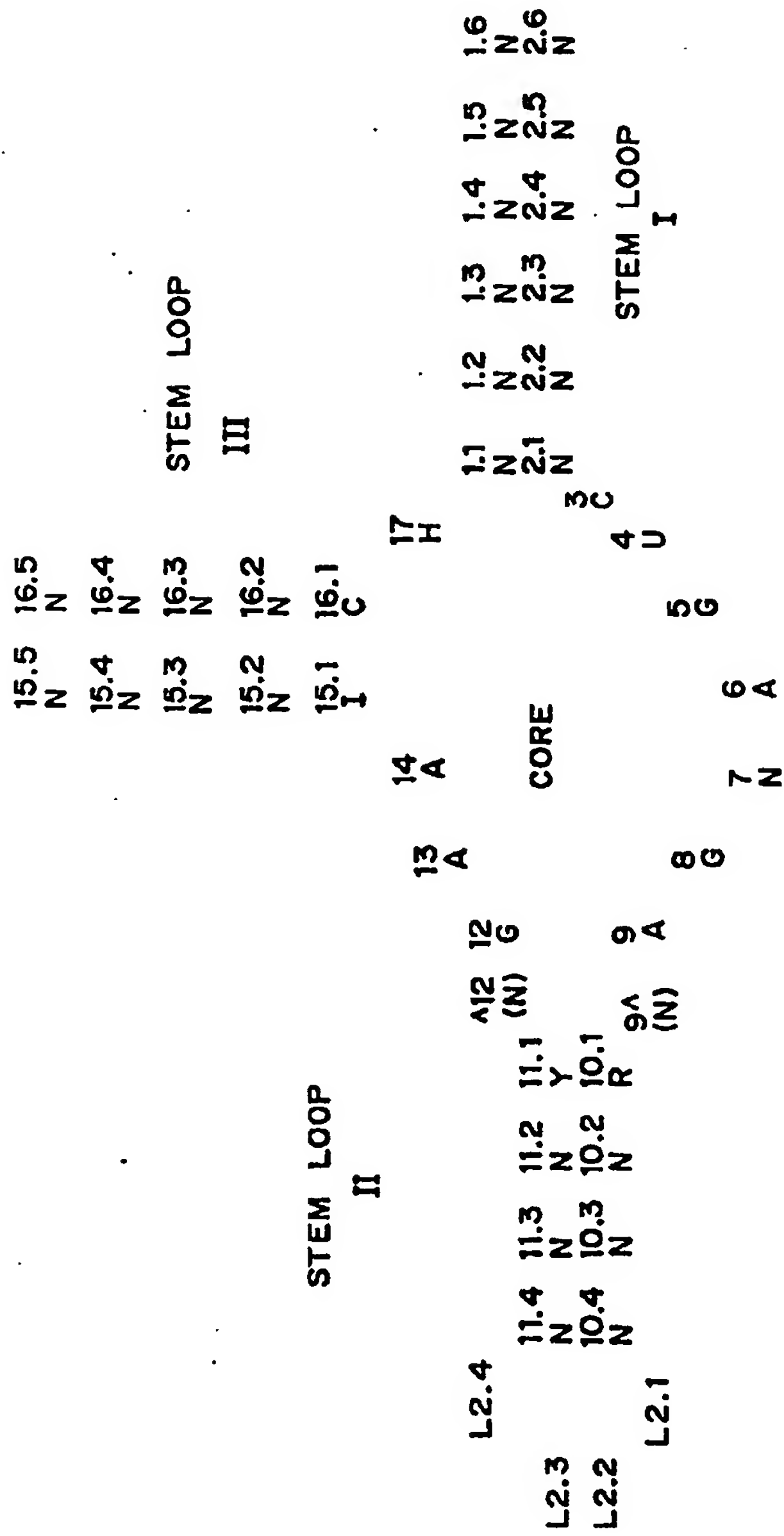


FIG. 2

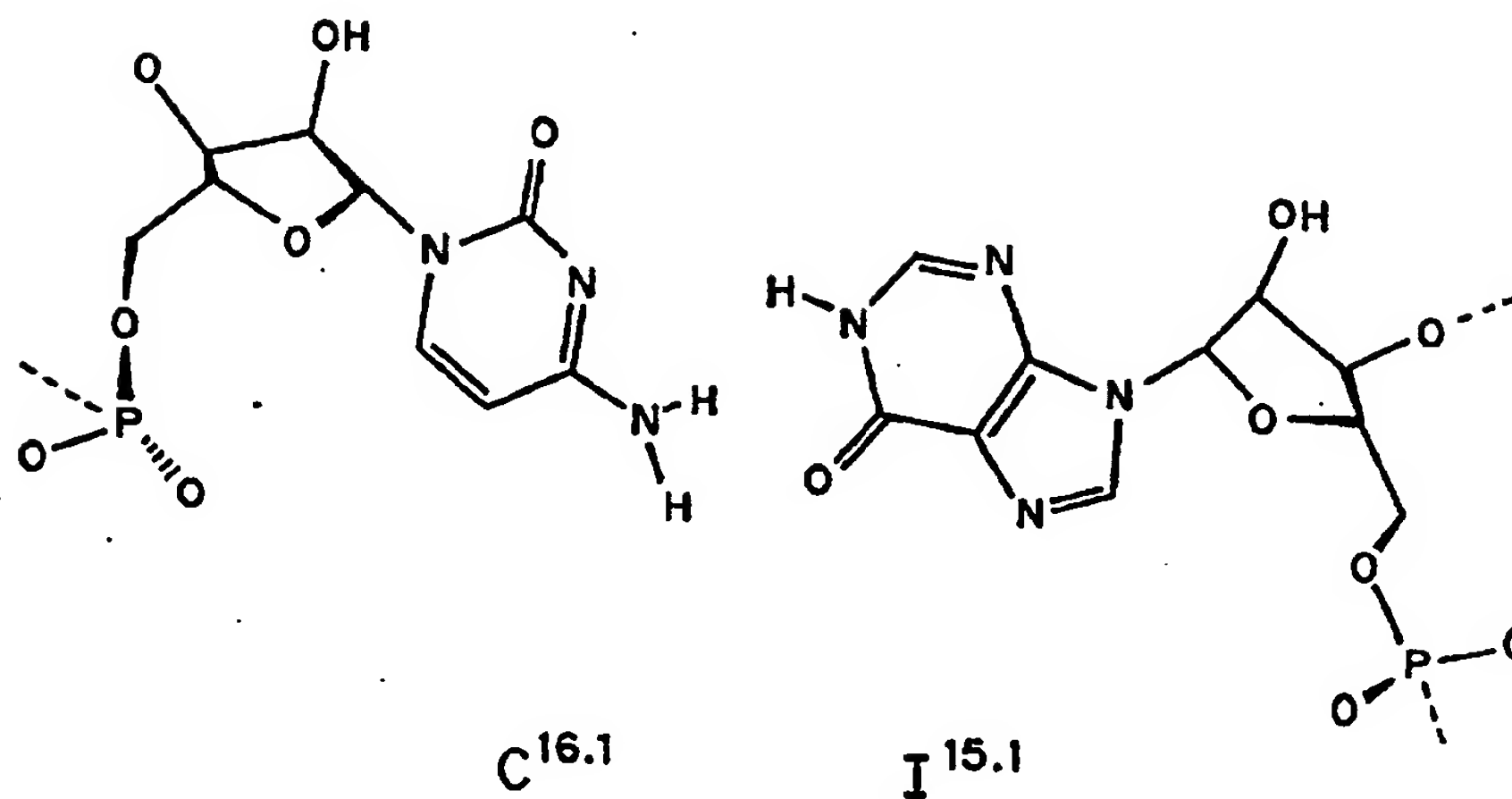
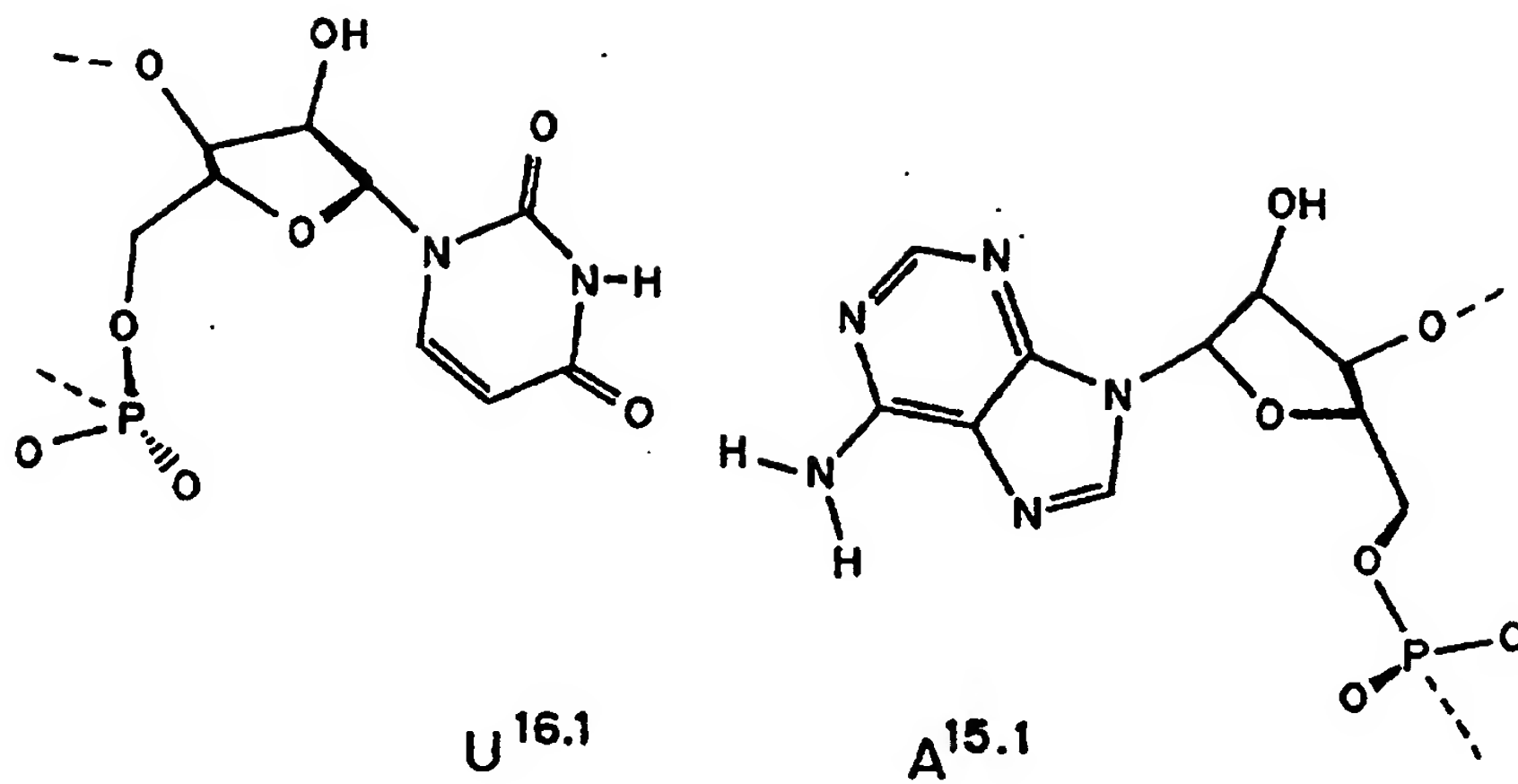


FIG. 3

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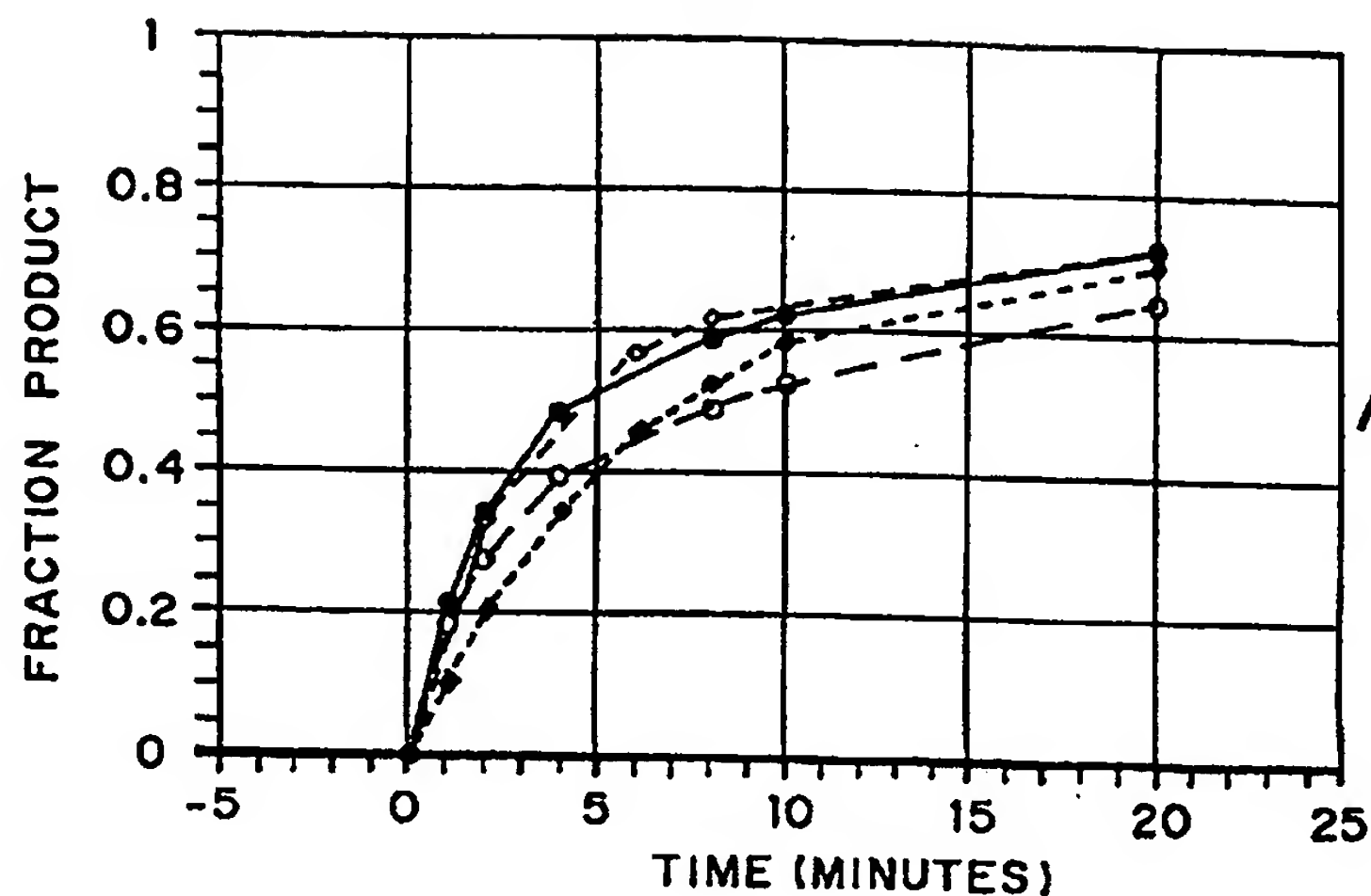


FIG. 4A

- N7 = 2'-allyloxy-2'-deoxyuridine
- N7 = 2'-allyloxy-2'-deoxycytidine
- ◇- N7 = 2'-allyloxy-2'-deoxyadenosine
- ◆- N7 = 2'-allyloxy-2'-deoxyguanosine

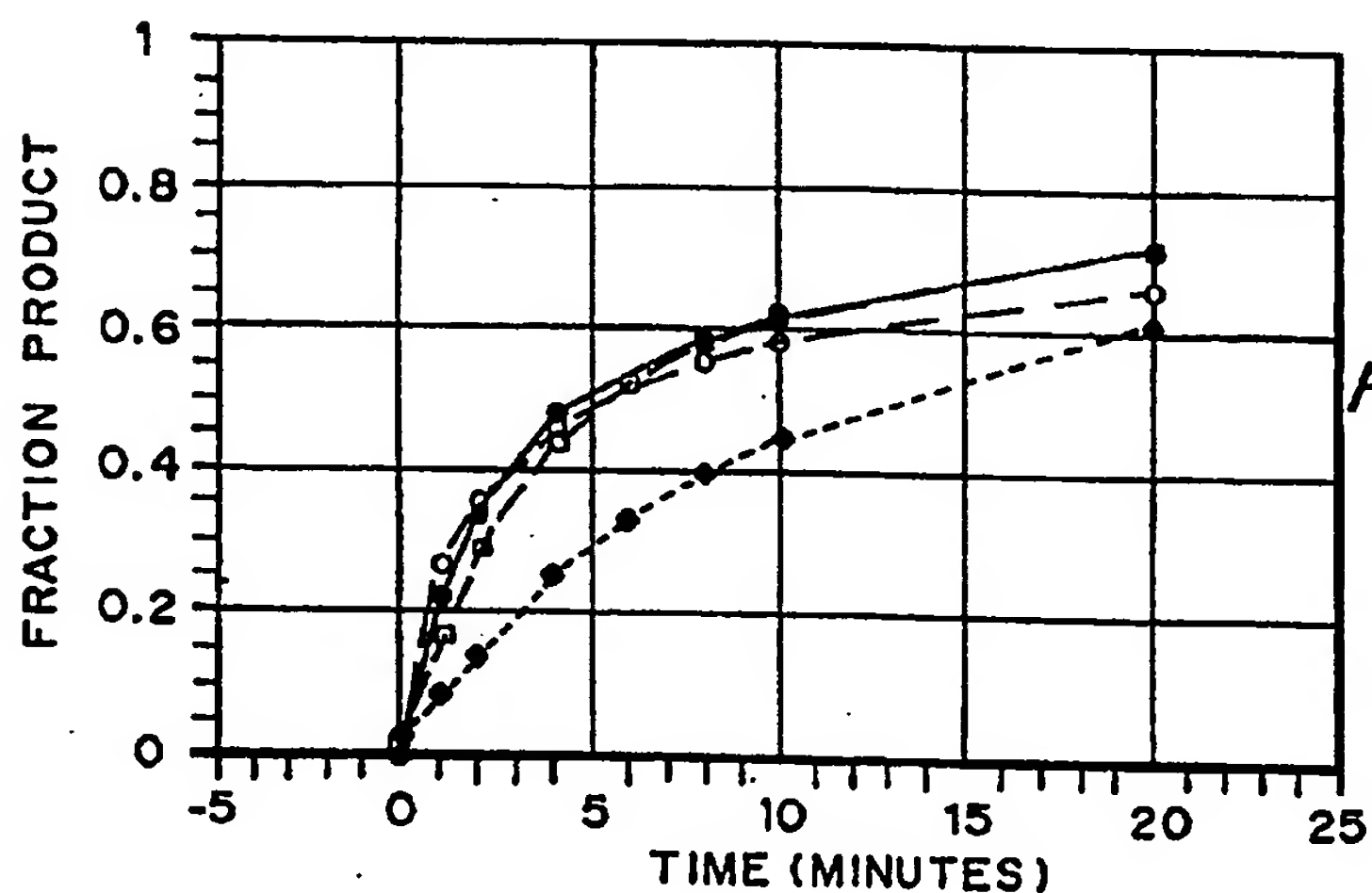


FIG. 4B

- N7 = 2'-allyloxy-2'-deoxyuridine
- ◆- N7 = 5-nitro-1-(2-O-allyl-β-D-ribofuranosyl) indole
- N7 = 2'-allyloxy-2'-deoxyinosine
- ◆- N7 = 1-(2-O-allyl-β-D-ribofuranosyl) quinazoline-2,4-dione

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INTERNATIONAL SEARCH REPORT

Int. National Application No.

PCT/US 98/12663

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/11 C12N9/00 C07H21/00 A61K31/70 //C1201/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 18312 A (VIMRX HOLDINGS LTD ;LUDWIG JANOS (DE); SPROAT BRIAN (DE)) 22 May 1997 cited in the application see page 4, line 15 - page 10, line 26 see claims see figures	1,3-23
A	PERRIMAN R ET AL: "EXTENDED TARGET-SITE SPECIFICITY FOR A HAMMERHAED RIBOZYME" GENE, vol. 113, no. 2, 1992, pages 157-163, XP000267805 cited in the application see pages 161-162, paragraphs (e) and (f)	1-23

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

12 November 1998

Date of mailing of the international search report

27/11/1998

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Authorized officer

Andres, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/12663

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AMONTOV S V ET AL: "HAMMERHEAD MINIZYMES WITH HIGH CLEAVAGE ACTIVITY: A DIMERIC STRUCTURE AS THE ACTIVE CONFORMATION OF MINIZYMES" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 118, no. 7, 21 February 1996, pages 1624-1628, XP002045193 cited in the application see the whole document	4-7
A	SINGH K K ET AL: "DESIGN OF HAMMERHEAD RIBOZYMES TO DISTINGUISH SINGLE BASE CHANGES IN SUBSTRATE RNA" ANTISENSE RESEARCH AND DEVELOPMENT, vol. 6, no. 3, 1996, pages 165-168, XP002020361 cited in the application see page 167, left-hand column, line 15 - line 25	1
A	SCOTT, W. ET AL.: "The crystal structure of an all-RNA hammerhead ribozyme: a proposed mechanism for RNA catalytic cleavage" CELL, vol. 81, 30 June 1995, pages 991-1002, XP002084034 cited in the application see page 995, left-hand column, line 16 - page 996, left-hand column, line 22	1
	ORTIGAO J F R ET AL: "ANTISENSE EFFECT OF OLIGODEOXYNUCLEOTIDES WITH INVERTED TERMINAL INTERNUCLEOTIDIC LINKAGES: A MINIMAL MODIFICATION PROTECTING AGAINST NUCLEOLYTIC DEGRADATION" ANTISENSE RESEARCH AND DEVELOPMENT, vol. 2, no. 2, 1992, pages 129-146, XP000573884 cited in the application	
P,X	LUDWIG J ET AL: "Extending the cleavage rules for the hammerhead ribozyme: mutating adenosine15.1 to inosine15.1 changes the cleavage site specificity from N16.2U16.1H17 to N16.2C16.1H17." NUCLEIC ACIDS RESEARCH, (1998 MAY 15) 26 (10) 2279-85., XP002084035 see the whole document	1-6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 12663

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/ US 98/12663

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark: Although claim 20 (as far as in vivo methods are concerned) and claims 22 and 23 are directed to a method of treatment of the human/animal body or to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Patent Application No.

PCT/US 98/12663

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9718312 A	22-05-1997	DE 19542404 A	15-05-1997
		AU 7572096 A	05-06-1997
		EP 0866865 A	30-09-1998

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